Gap Junction–Mediated Import of MicroRNA from Bone Marrow Stromal Cells Can Elicit Cell Cycle Quiescence in Breast Cancer Cells

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Abstract

Bone marrow (BM) metastasis of breast cancer (BC) can recur even decades after initial diagnosis and treatment, implying the long-term survival of disseminated cancer cells in a dormant state. Here we investigated the role of microRNAs (miRNA) transmitted from BM stroma to BC cells via gap junctions and exosomes in tumor cell quiescence. MDA-MB-231 and T47D BC cells arrest in G0 phase of the cell cycle when cocultured with BM stroma. Analyses of miRNA expression profiles identified numerous miRNAs implicated in cell proliferation including miR-127, -197, -222, and -223 targeting CXCL12. Subsequently, we showed that these CXCL12-specific miRNAs are transported from BM stroma to BC cells via gap junctions, leading to reduced CXCL12 levels and decreased proliferation. Stroma-derived exosomes containing miRNAs also contributed to BC cell quiescence, although to a lesser degree than miRNAs transmitted via gap junctions. This study shows that the transfer of miRNAs from BM stroma to BC cells might play a role in the dormancy of BM metastases.

Introduction

Breast cancer (BC) remains a clinical dilemma despite education, aggressive intervention, and early detection (1). A significant number of BC recurrences have identified bone marrow (BM) as the source of cancer cells (2–4), supporting the existence of dormant BC cells (BCC). BC resurgence has occurred without a history of primary tumor (5). Recent mathematical models indicate dormancy by a small number of BCCs with half-life of more than 12 years (6).

Metastasis of BC to BM correlates with poor prognosis (7, 8). BC could resurface from BM after more than 10 years of remission, in the absence of hematopoietic disruption (9–11). BCCs in BM would be best targeted if there is an understanding of how BCCs coexist within the microenvironment of BM. These form gap junctional intercellular communication (GJIC) with BM stroma (11), which comprise the hematopoietic niche close to the endosteum (12–19). The location of dormant BCCs in this area of BM might explain why high-dose chemotherapy and autologous stem transplantation failed (9, 20).

Connexin 43 (Cx43) contributes to the formation of GJIC between BCCs and stroma (11). CXCL12 production is decreased in BCCs when they contact stroma, and this is linked to reduced proliferation of BCCs as well as normal hematopoietic function (11). It is presumed that the latter occurs because CXCR4-expressing hematopoietic stem cells (HSC), instead of BCCs, interact with CXCL12-expressing stroma. This study investigates the role of specific microRNAs (miRNA) in the quiescence of BCCs in contact with stroma. miRNAs have been implicated in BC metastasis and to particular secondary organs (21–24).

CXCL12 belongs to the chemokine family and interacts with CXCR4 and CXCR7 (25). It is ubiquitously expressed and is constitutively produced in BM stroma (25, 26). miRNAs are small (19–23 nucleotide) noncoding RNA (27) that mostly target the 3′-untranslated region (UTR) of mRNAs with imperfect homology (27, 28). In this study, we show exchange of miRNAs across GJIC between BCCs and BM stroma and that specific miRNAs target CXCL12 to reduce the proliferation of BCCs.

Materials and Methods

Reagents

1-Octanol and protein G Sepharose were purchased from Sigma; FCS from Hyclone Laboratories; Platinum SYBR Green
SuperMix and SuperScript III from Invitrogen (www.invitrogen.com); Western Blot Stripping Buffer from Thermo Scientific; propidium iodide (PI) from BD Biosciences; RNase A from Qiagen; and CXCL12 ELISA kit from R&D Systems.

**Antibodies**

Secondary antibodies [fluorescein isothiocyanate (FITC) and phycoerythrin conjugated], rabbit anti-human CXCRI7, and rabbit anti-cyclin C were purchased from Abcam; murine anti-cyclin D1 and CDK from Cell Signaling; mouse anti-cytokeratin and mouse anti-β-actin monoclonal antibodies (mAb) from Sigma; murine anti-human CXCL12 mAb from R&D Systems; horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG from Santa Cruz Biotechnology; and rat anti-CD63/LAMP-3 and rabbit anti-acetyl histone H3 from Upstate Cell Signaling.

**Cells**

MDA-MB-231 and T47D were purchased from the American Type Culture Collection (ATCC) and propagated according to their instructions. Stroma was prepared from BM aspirates of healthy donors, 18 to 25 years of age, as described (29). The protocol was approved by the Institutional Review Board of University of Medicine and Dentistry of New Jersey. At confluence, adherent cells were subcultured in 10% FCS and after 3 passages were negative for CD14 and more than 99% positive for prolyl-4-hydroxylase.

Cocultures of BCCs and BM stroma were described previously (30). Briefly, BCCs and stroma were cocultured at equal ratios in α-MEM with 10% FCS, in the presence or absence of 300 μmol/L 1-octanol (31).

**Cell separation**

Cells were deadhered with accutase, resuspended at 10⁷/mL in PBS with 2% FCS and anti-cytokeratin (1/1,000), and then incubated at 0 °C for 1 hour. After this, the cells were positively selected with Dynabeads M-450 goat anti-mouse IgG (Invitrogen). The negatively selected cells were stroma. BCCs were detatched with DETACHaBEAD (Invitrogen). Flow cytometry analyses indicated more than 95% cytokeratin (+) for the Dynabead fraction and more than 99% prolyl-4-hydroxylase (+) for the stromal fraction.

**Western analyses**

Whole cell extracts were prepared and analyzed by Western blot, as described (32). Nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific). CXCR7 was studied with membrane extracts, which was prepared with Qproteome plasma membrane protein extraction kit (Qiagen). Blots were developed overnight with anti-CXCL12 or anti-CXCR7 (at 1/1,000) and anti-cyclin C, anti-p21, and anti-cyclin D3 (at 1/500). The primary antibody was detected with HRP–anti-mouse IgG at 1/2,000 (PerkinElmer). Anti-histone H3 and β-actin were used at 1/1,000 and 1/4,000, respectively.

**Real-time reverse transcriptase PCR for miRNAs**

Predicted miRNAs for the 3'-UTR of CXCL12 were determined with miRNAplus algorithm and then verified by real-time reverse transcriptase PCR (RT-PCR), as described (33), with primers from Ambion with SS rRNA as the normalized values. The miRNA array was done with total RNA from T47D and stroma that were cultured alone and in cocultures. RNA was sent to Rutgers University (New Brunswick, NJ) for analyses with the ABI TaqMan miRNA v1.0.

**Vectors, pre-miRs, and anti-miRs**

pMIR-R/Tac1/SG has been described (33). pCMV-DsRed-Express2 was purchased from Clontech. pEZX-MT01 reporter gene with the 3'-UTR of CXCL12 (hereafter pEZX-CXCL12) was purchased from Genescopea. All pre-miRs and anti-miRs were purchased from Ambion.

**Antisense exchange in cocultures**

BCCs were transfected with pCMV-DsRed-Express2 and then selected with 600 μg/mL G418. After this, cells with bright red fluorescence (RFP) were sorted and then established as cocultures with stroma, as described (30, 34). The stromal cells were transiently transfected with 20 μmol/L of fluorescein-labeled antisense dsRED or scrambled sequence (Sigma), using Effectene (Qiagen). The antisense 5' acc atg gat act gac gac g 3' was complementary to the region spanning the translation start site of the DsRed mRNA. The 3' end of the antisense was tagged with fluorescein (green) to track the passage into BCCs. After 48 hours, the passage of the antisense was confirmed by flow cytometry that detects FITC (not shown). The involvement of GJIC was determined in parallel cocultures with 300 μmol/L of 1-octanol.

**Reporter gene assay**

Reporter gene assays was described previously (33). Briefly, BCCs were transfected with pMIR-R/Tac1/SG with FuGENE and, after 12 hours, retransfected with miR-130a and/or miR-206, using siPORT NeoFX (Ambion). After 16 hours, cell-free lysates were analyzed for total protein (Bio-Rad) and luciferase (Promega). Luciferase was normalized with β-gal activity and presented as normalized luciferase per microgram of total protein. Luciferase activity was determined in cotransfections (16 hours) of T47D with pEZX-CXCL12 and pre-miR-127, -197, -227, and/or -223.

**Proliferation/viability assessment**

Proliferation and viability were assessed in cocultures of T47D and stroma with CyQuant Cell Proliferation Assay Kit (Molecular Probes) and CellTiter-Blue Cell Viability Assay Kit (Promega), respectively. 

Day 1: T47D was transfected with the anti-miR or control anti-miR, using siPORT NeoFX. Day 2: Media were removed and the transfectants were established as cocultures. Day 3: Since GJIC requires ~24 hours (11), proliferation was determined after 3 days of GJIC (day 6). Proliferation was calculated from a standard curve of T47D and stroma. Parallel studies were done with stroma alone, and the values were subtracted from total proliferation. The percent viability was calculated from a standard curve with untreated healthy cells assigned 100% viability. Cell-free wells with reagent alone were assigned 0% viability.
Cell cycle analyses

Cell cycle analyses were studied by PI incorporation and also by double labeling with dyes Pyronin Y and Hoechst 33342. In the former analysis, cocultures were incubated with anti-cytokeratin and FITC–anti-IgG secondary antibodies to label BCCs. Controls were labeled with nonimmune isotype or FITC–anti-IgG. In the case of PI labeling, the cells were treated with RNase A (1 mg/mL) and fixed with 70% ethanol and then labeled with 20 μg/mL PI solution. G0 and G1 phases of the cell cycle were distinguished by the consecutive addition of Hoechst 33342 (5 μg/mL) at 37°C for 45 minutes and 1 μg/mL of Pyronin Y (Sigma) for 15 minutes at 37°C. Flow cytometric analyses gated the cytokeratin (+) cells, at emissions 575 nm (Pyronin Y) and 450 nm (Hoechst 33342), and the data were analyzed with BD CellQuest software.

Data analyses

Statistical analyses were done with ANOVA and the Tukey-Kramer multiple comparisons test. P < 0.05 was considered significant. The predicted functional networks of miRNA in T47D from cocultures with stroma were generated by analyzing the validated and predicted targets with Ingenuity Pathways Analysis (IPA; Ingenuity Systems; www.ingenuity.com).

Results

Cell cycle quiescence of BCCs in cocultures with stroma

BCC proliferation is decreased upon contact with BM stroma (11). This study examined T47D and MDA-MB-231 for cell cycle proteins in time-course studies with nuclear extracts. We observed time line decreases in cyclins D3 and C but increased p21 (Fig. 1A). The first time point, 12 hours, was selected to coincide with GJIC (11). Since the Western blots required robust manipulation, we asked the same questions without subjecting the cells to selection processes. We colabeled the cocultures (n = 4) with PI and anti-cytokeratin to identify BCCs and then analyzed the cells by flow cytometry. Cocultured T47D showed significant (P < 0.05) increase in G0/G1 phase (Fig. 1C and D). These findings correlated with light bands for cyclin D1 and CDK4 (Fig. 1E). We next discriminated G0 from G1 phase with Pyronin Y and Hoechst dye and observed more than 80% T47D in G0 phase (Fig. 1F).

Passage of antisense DsRed via GJIC

We previously showed GJIC between BCCs and BM stroma by dye exchange, which correlated with a decrease in the proliferation of BCCs (11). In order to study the role of GJIC in BCC quiescence, we asked if small RNA can pass through GJIC, using antisense oligos. T47D and MDA-MB-231 were stably transfected with pCMV-DsRed-Express2 (Fig. 1G) and then cocultured with stroma that was transfected with antisense or scrambled oligo DsRED. In 3 experiments (Fig. 1H), we analyzed the fluorescence of DsRED at 200× magnification with EVOS digital microscope (Advanced Microscopy Group). BCCs that contacted stroma resulted in dim to undetectable fluorescence. Similar change was not observed for scrambled oligos or, with GJIC inhibitor, 1-octanol or carbenoxolone (not shown). In summary, the results indicated that antisense DsRed oligos can pass from stroma to BCCs through GJIC.

Passage of miRNA via GJIC between BCCs and stroma

In this set of studies, we investigated if miRNAs can pass through GJIC between BCCs and stroma. We addressed this question with a system in which the miRNAs (−130a and −206) were known to suppress luciferase in pMiR-R/Tac1/SG (33). The pre-miRs were transfected in stroma and pMiR-/Tac1/SG, in BCCs. After 48 hours, luciferase levels were significantly (P < 0.05) less than negative control pre-miR (Fig. 2A and B). This effect was reversed with 1-octanol. The results of both cell lines are combined in the appropriate experimental point (Fig. 2C). The decrease in luciferase by 1-octanol was not caused by degraded pre-miRs since real-time PCR showed no change in their levels (Fig. 2D).

miRNA levels in T47D and stroma before and after coculture

We focused on T47D to investigate miRNA passage from stroma to BCCs. The first set of studies analyzed miRNA profile in T47D and stroma, before and after coculture, using ABI TaqMan human miRNA arrays (v1.0). The raw cycle threshold (Ct) values before and after coculture are shown in Figure 3A. The circled points indicate low expression before coculture (high Ct values) and high expression after coculture (low Ct values). The increased miRs in stroma suggested that they were induced after contact with T47D.

We next normalized the Ct values of all miRNAs to one of the internal controls (RNU48) in the ABI array (ΔCt). The ΔCt values before coculture were subtracted from the ΔCt after coculture (ΔΔCt) and those with more than 25-fold increases are presented in Figure 3B. The miRNAs levels in T47D and stroma of cocultures were reciprocal, suggesting their movement from stroma to T47D. IPAs of literature-validated targets studied the predicted functions of the miRNAs (Fig. 3B) and their network (Fig. 3C). We next selected those that showed more than 10-fold change and then submitted to IPAs. We selected the top 3 of 9 networks and edited them to focus on relationships associated with differentiation (Fig. 3D). On the basis of the data (Fig. 3C and D), we deduced that the increases in miRNAs in T47D were linked to cell cycle quiescence and functions and blunted cell differentiation.

We next determined whether exosomes, produced from stroma, contributed to cell cycle quiescence of T47D. We collected stroma-conditioned media during a 48-hour period and prepared exosome-free and exosome-containing media (method in Supplementary S1). The 2 sets of media were added to T47D, and after 48 hours we studied cell cycle analyses with PI. There was a trend toward G0/S phase with the exosome-free conditioned media (Fig. 3E and F). The presence of exosomes was confirmed by immuno-precipitation with anti-CD63 (Fig. 3F inset) and Western blot (not shown). In summary, the studies suggested that stroma-derived exosomes could have a minor role in T47D quiescence.
Figure 1. Cell cycle analyses of BCCs and passage of DsRed in cocultures. Western blots ($n = 4$) were done for cell cycle proteins in T47D (A) and MDA-MB-231 (B). Cell cycle analyses of cocultured T47D ($n = 4$) before (C) and after 36 hours (D). Western blots ($n = 3$) for cyclin D1 and CDK4 with T47D nuclear extracts of coculture before and after 36 hours (E). Pyronin Y/Hoechst labeling of T47D alone or 36-hour coculture (F). Fluorescence-sorted T47D or MDA-MB-231, stably transfected with pCMV-DsRed-Express2 (G), were established as coculture with stroma. The stroma was transfected with antisense or scrambled DsRed oligonucleotides (H) in the presence or absence of 300 μmol/L 1-octanol. Images are shown at 200× with arrows indicating the loss of RFP when BCCs contact stroma.
Changes in CXCL12-predicted miRNAs

The section focused on CXCL12 miRs in mechanistic studies to understand cell cycle quiescence. We previously showed a decrease in CXCL12 in BCCs after contact with stroma (11). miRanda algorithm (35) predicted clusters of miR-127, -197, -222, and -223 at several sites within the 3' UTR of CXCL12 (Fig. 4A). Real-time PCR indicated high levels of miR-127, -222, and -223 in stroma but low to undetectable in T47D (Fig. 4B). After cocultures, all 4 miRs were significantly (P < 0.05) increased in T47D (open bars) as compared with in stroma (diagonal bars; Fig. 4C). These increases were reversed with 300 μmol/L 1-octanol (Fig. 4D, solid bars) but remained at high levels in stroma, except for miR-197 (Fig. 4D, diagonal bars). These results indicated that CXCL12-predicted miRNA can pass from stroma to T47D via GJIC.

Validating the predicted CXCL12 miRNAs

We studied the specificity of the predicted CXCL12 miRNA, using a reporter gene vector (pEZX-CXCL12) with the 3'-UTR of CXCL12, downstream of luciferase. T47D were transfected with pEZX-CXCL12 and/or pre-miR-127, -197, -222, or -223. Each pre-miR resulted in significant (P < 0.05) decrease in luciferase as compared with negative control pre-miRs (Fig. 5A). Pre-miR-197 caused significant (P < 0.05) reduction in luciferase as compared with the other pre-miRs (Fig. 5A).

We next determined if luciferase activity could be similarly decreased if the pre-miRs were transfected in stroma and pEZX-CXCL12 in T47D. Indeed, after 48 hours of coculture, luciferase levels were significantly (P < 0.05) decreased as compared with negative control miR (Fig. 5B). This indicated that the miRNAs passed from stroma to T47D. We next asked if the changes in luciferase could be caused by stroma-derived factors. We therefore placed pEZX-CXCL12-transfected stroma in fresh or stroma-conditioned media and observed similar luciferase activity: 2,426 ± 44 versus 2,421 ± 25 normalized luciferase, n = 3.

Because of predicted overlap of the miRNAs in the 3'-UTR of CXCL12 (Fig. 5C), we asked if combinations of pre-miRs can further decrease luciferase. The results showed significant (P < 0.05) reduction by more than 1 pre-miRs as compared with negative control pre-miR (Fig. 5D) and single transfectants (Fig. 5A). miR-197 was most efficient in reducing luciferase when combined with pre-miR-222 and pre-miR-223.
Figure 3. Pathway analyses of miRNA array. TaqMan miRNA array was done with T47D and stroma before and after cocultures (Cts). The changes in miRNA were analyzed with Ingenuity Pathway program (A). T47D with more than 25-fold increase after cocultures were plotted as a histogram with the corresponding values in stroma (B). T47D with more than 10-fold increase in coculture were uploaded into the Path Designer graphical application and then overlaid onto a global molecular network developed from information contained in Ingenuity’s knowledge base. The image shows direct, experimentally determined relationships between miRNA and other molecules (C and D). Cell cycle analyses of T47D after culture for 48 hours with exosome-free stroma-conditioned media (E) or cultured with exosome-containing media (F). F, inset (i), immunoprecipitation with anti-CD63.
We further validated miR-127, -197, -222, and -223 for specificity to CXCL12 in Western blots with plasma membrane extracts from cocultured T47D and stroma. The results indicated undetectable band for T47D and a bright band when GJIC was interrupted with 300 μmol/L of 1-octanol (Fig. 5E).

We next determined if the decrease in membrane-bound CXCL12 correlated with released protein. We knocked down the miRNAs in T47D with anti-miRs or further expressed them with pre-miRs and then established the T47D as cocultures with stroma. After 48 hours, the media were quantitated for CXCL12 by ELISA. The results showed significant (P < 0.05) increase when miRNAs were knocked down as compared with control anti-miR and untransfected cells (Table 1). There were similar or reduced CXCL12 levels by pre-miR as compared with untransfected T47D or control pre-miR (Table 1).

Although CXCR4, the receptor for CXCL12, is expressed on BCCs (36), CXCL12 can also interact with CXCR7 (37). This led us to investigate if CXCR7 was expressed on BCCs since this could make the cells more responsive to CXCL12, or act as a decoy by interacting with CXCL12, away from CXCR4 to prevent signaling. Western blot analyses showed CXCR7 on T47D and MDA-MB-231 (Fig. 5F).
reduced proliferation as compared with negative control pre-miR (Fig. 6A). In "loss of function" studies, T47D was transfected with anti-miRs, and the results indicated significant (P < 0.05) increase in T47D proliferation as compared with negative miRNA and untransfected T47D (Fig. 6B). There was no change in cell viability of transfec-
tants. The proliferation was blunted by 1-octanol (not shown). In summary, miR-127, -197, -222, and -223 caused T47D proliferation in coculture with stroma.

The next set of studies investigated if the miRNAs can cause off-target effects in the proliferation of T47D. We stably knocked down CXCL12 with wild-type (wt) or mutant (mut) siRNA and then transfected the cells with pre-miR or negative control pre-miR. As expected, after 48 hours, there was significantly (P < 0.05) reduced cell proliferation with wt siRNA as compared with mut siRNA (Fig. 6C). There was no off-target effect by miR-197, miR127, and miR-222 in the knockdown cells, since the outcome was similar to mut siRNA and pre-miR transfectants (Fig. 6C).

Since the miRNAs shown in Figure 6A–C indicated roles for BCC quiescence, we determined if quiescence might involve other genes. IPA of a network with miR-127, -222, and -223 were established with predicted and validated targets. The addition of tags to highlight the molecules involved in cell cycle supported the roles of the miRs in cell cycle quiescence.
miR-197 did not connect to the other miRNAs, but alone it was associated with cell cycle quiescence, proliferation, and growth (Fig. 6E).

To verify that the decrease in CXCL12 and GJIC caused cell cycle quiescence of cocultured T47D, we undertook 48-hour cocultures in the presence of 300 μmol/L 1-octanol or 1 ng/mL CXCL12 (G).

Figure 6. CXCL12 miRNAs in the proliferation of cocultured T47D. Cell count of cocultures with T47D, transfected with test or control (Neg) pre-miR, mean ± SD, n = 4 (A). Similar studies (n = 4) with anti-miR transfectants; P < 0.05 versus anti-miR (B). CXCL12 knockdown T47D (wt) or mut siRNA were transfected with test or negative control pre-miR. After 48 hours, cell proliferation was assessed (n = 4) and the data presented as above, *P < 0.05 versus wt siRNA; **, P < 0.05 versus Neg pre-miR (C). miRNAs were analyzed with the Ingenuity Pathway program for predicted and validated targets for miR-127, miR-222, and miR-223 (D) and miR197 for predicted targets (E). Cell cycle analyses (n = 3) were done in 48-hour cocultures with T47D, in the presence of 300 μmol/L 1-octanol (F) or 1 ng/mL CXCL12 (G). Each experiment used stroma from a different donor. *, P < 0.05 versus anti-miR.
CXCL12. The latter was within the range of CXCL12, produced from untransfected T47D and anti-miR-treated T47D (Table 1). The coculture cells were deadhered with accutase and then colabeled with PI and FITC–anti-cytokeratin. While untreated T47D was mostly in the G1 phase (Fig. 1D), treatment with 1-octanol and CXCL12 caused cycling (Fig. 6F and G).

Discussion

BCCs close to the endosteum of mice have long doubling times (34), consistent with dormant BCCs in BM (7, 8). We showed a role for miRNAs in BCC quiescence by demonstrating their passage through GJIC between BCCs and BM stroma. The passage of small nucleotides was shown, first with antisense and then with miRNA (Fig. 1G and H and 2). Array analyses indicated that miRNAs were significantly increased in cocultured T47D with concomitant decreases in stroma (Fig. 3A and B). This suggested that the miRNAs moved from stroma through GJIC to T47D (Fig. 3B). IPAs with validated and predicted functions indicated that the miRNAs could be involved in cell cycle quiescence (Fig. 3C and D). This prediction was expected because of the association of miRNAs with tumor suppressive and oncogenic functions (38). We identified 4 miRNAs that suppressed CXCL12 production in cocultured T47D to decrease cell proliferation and transitioned the T47D to G0 phase of the cell cycle. These findings explained our previous study that showed long doubling time by BCCs close to the endosteum of nude mice (34) and also explained why CXCL12 was decreased in coculture (11). The decreased CXCL12 in BCCs was relevant to BM homeostasis and also provided BCCs with an advantage to adapt dormancy. A decrease in CXCL12 prevents CXCR4-expressing BCCs from movement of CXCL12-specific miRNAs to BCCs for a decrease in proliferation and CXCL12 production. The cartoon also depicts the exchange of additional miRNAs with unidentified functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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