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Essential role for TrpC5-containing extracellular vesicles in breast cancer with chemotherapeutic resistance

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A critical challenge for chemotherapy is the development of chemoresistance in breast cancer. However, the underlying mechanisms and validated predictors remain unclear. Extracellular vesicles (EVs) have gained attention as potential means for cancer cells to share intracellular contents. In adriamycin-resistant human breast cancer cells (MCF-7/ADM), we analyzed the role of transient receptor potential channel 5 (TrpC5) in EV formation and transfer as well as the diagnostic implications. Up-regulated TrpC5, accumulated in EVs, is responsible for EV formation and trapping of adriamycin (ADM) in EVs. EV-mediated intercellular transfer of TrpC5 allowed recipient cells to acquire TrpC5, consequently stimulating multidrug efflux transporter P-glycoprotein production through a Ca^{2+}- and activated T-cells isoform c3-mediated mechanism and thus, conferring chemoresistance on nonresistant cells. TrpC5-containing circulating EVs were detected in nude mice bearing MCF-7/ADM tumor xenografts, and the level was lower after TrpC5–siRNA treatment. In breast cancer patients who underwent chemotherapy, TrpC5 expression in the tumor was significantly higher in patients with progressive or stable disease than in patients with a partial or complete response. TrpC5-containing circulating EVs were found in peripheral blood from patients who underwent chemotherapy but not patients without chemotherapy. Taken together, we found that TrpC5-containing circulating EVs may transfer chemoresistance property to nonchemoresistant recipient cells. It may be worthwhile to further explore the potential of using TrpC5-containing EVs as a diagnostic biomarker for chemoresistant breast cancer.

The development of chemotherapeutic resistance in breast cancer is a serious problem (1, 2). To date, the mechanisms underlying chemoresistance are still largely unknown, and no validated predictive factor of chemoresistance is available in the clinic. Therefore, it is important to identify the signaling pathways and search for circulating markers in breast cancer resistant to chemotherapy.

The extracellular environment contains a large number of mobile membrane-limited vesicles named extracellular vesicles (EVs). Major EV populations include exosomes, microvesicles, and apoptotic bodies (1, 3–5). These dynamic EVs may have essential function in intercellular communication and immune regulation (5). Tumor cells also generate EVs (3, 4). Large quantities of tumor-derived circulating EVs have been found in the blood of patients with glioblastoma multiforme (4), pancreatic cancer (6), gastric cancer (7), and acute myeloid leukemia (8). They contain cell surface proteins, RNA, and DNA (3, 4, 9, 10). They mediate intercellular cross-talk by transferring their intravesicular contents from donor to recipient cells and participating in tumor invasion and metastasis (11–13). However, how these structures are generated and their importance in chemotherapeutic resistance in breast cancer are poorly understood.

On the basis of our previous finding that transient receptor potential channel 5 (TrpC5) regulates the multidrug transporter P-glycoprotein (P-gp) (13), we investigated the possible role of TrpC5 in the formation and release of EVs in the context of chemotherapeutic drugs. TrpC5 is one of the mammalian transient receptor potential proteins. It is a nonselective cation channel with Ca^{2+} permeability (14). Functionally, TrpC5 is involved in growth factor-regulated vesicular trafficking through PI3K, Rac1, and PIP-5-kinase–mediated pathways (15). Here, we describe findings that suggest a unique role for TrpC5 in transfer of chemoresistance and its diagnostic implications.

Significance

A critical challenge for chemotherapy is development of chemoresistance, but underlying molecular mechanisms remain unclear. In this study, we found that drug-resistant adriamycin-resistant human breast cancer cells possessed numerous transient receptor potential channel 5 (TrpC5) -containing extracellular vesicles (EVs) on the cell surface. Suppressing TrpC5 expression diminished the formation of EVs. Incubation of drug-sensitive recipient cells with EVs endowed recipients with drug-resistant properties. In both human samples and a mouse model of breast cancer, the expression of TrpC5 proteins was high in the tumor, and the levels of TrpC5-positive EVs were high in the circulation. These data suggest a critical role of TrpC5-containing EVs in the transfer of drug resistance. In the future, monitoring TrpC5-containing EVs in the circulation could potentially be used to predict the clinical outcome of chemotherapy.

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Results

TrpC5 Is Required for EV Formation in Adriamycin-Resistant Human Breast Cancer Cells. We established adriamycin-resistant human breast cancer cells (MCF-7/ADM). These cells, with greatly up-regulated multidrug efflux transporter P-gp (13), displayed a 630-fold greater resistance to adriamycin (ADM) than the parental line MCF-7/WT. They also displayed enhanced resistance to paclitaxel (146-fold), epirubicin (56-fold), vincristine (5-fold), and mitoxantrone (127-fold).

Interestingly, we found a great enhancement of EV structures on the surface of MCF-7/ADM cells by transmission EM (TEM) (Fig. 1*A*) compared with the WT (Fig. 1*B*). ADM has natural red fluorescence, and it functions by intercalating into DNA (16). In MCF-7/WT cells, as expected, ADM mainly accumulated inside the nucleus, which was shown by confocal microscopy (red autofluorescence) (Fig. 1*C*). However, in MCF-7/ADM cells, ADM accumulation was much less, with most of the residual drug in the periphery and not in the nucleus (Fig. 1*C*). Immuno-TEM and an ADM-specific monoclonal antibody were further used to verify its accumulation in EVs. Images clearly showed an EV distribution of ADM (Fig. 1*D*). Immunostaining for the EV marker flotillin2 (17) in intact MCF-7/ADM cells confirmed the identity of puncta as EVs (Fig. 1*E*). These results clearly indicated that ADM was trapped in EVs (Fig. 1*E*), where its pharmacological effect would be reduced.

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) is an important signal for EV formation (18–20), whereas TrpC5 is a Ca^{2+}-permeable channel (21, 22) that is involved in local vesicular trafficking (15) with substantially higher expression in chemoresistant MCF-7/ADM cells (21). Thus, we further tested whether TrpC5 is involved in the formation of EVs. In chemoresistant MCF-7/ADM cells, immunostaining as well as immuno-TEM clearly showed the accumulation of TrpC5 in EVs (Fig. 1*F*). Immunostaining showed that TrpC5 is colocalized with flotillin2-positive EVs

Fig. 1. TrpC5 is required for EV formation in MCF-7/ADM cells. (*A* and *B*) Transmission electron micrographs of (*A*) ADM-resistant (MCF-7/ADM) and (*B*) ADM-sensitive (MCF-7/WT) human breast cancer cells. (*C*) Confocal immunostaining micrographs of ADM (autofluorescence; red) distribution in EVs in MCF-7/ADM and MCF-7/WT cells. DIC, differential interference contrast. (*D*) Immunotransmission electron micrograph of ADM distribution (arrows) in EVs. (*E*) Confocal immunostaining micrograph of flotillin2 expression in EVs from MCF-7/ADM cells. (*F*) Confocal immunostaining micrographs (*Left*) and immunotransmission electron micrograph (arrows; *Right*) showing distribution of TrpC5 in EVs. (*G*) Transmission electron micrographs showing effects of TrpC5 knockdown on EV formation in MCF-7/ADM cells (*Left*, scrambled; *Right*, TrpC5-siRNA). (*H*) Confocal immunostaining micrographs showing the effect of TrpC5 knockdown on ADM distribution in EVs in MCF-7/ADM cells (*Left*, scrambled; *Right*, TrpC5-siRNA). TrpC5-siRNA or scrambled siRNA (200 nM); ADM (10 μg/mL; overnight) was added to the culture medium 1 d before fluorescence measurement. (Scale bars: 1 μm.)

near cell periphery (Fig. S1). Up-regulated P-gp (21), which is coded by the *mdr1* gene and pumps cytotoxic drugs from MCF-7/ADM cells, also had an EV distribution (Fig. S2). In MCF-7/ADM cells, TEM showed that TrpC5-specific siRNA greatly inhibited the generation of EVs compared with scrambled siRNA by TEM (Fig. 1*G*), and confocal microscopy showed that ADM reaccumulated in the nucleus (Fig. 1*H*). We further constructed an MCF-7 cell line stably expressing TrpC5 and found that up-regulation of TrpC5 greatly increased the generation of EVs (Fig. S3). These results showed that TrpC5 plays an essential role in EVs formation by chemoresistant breast cancer cells.

TrpC5-Containing Circulating EVs Are Released from Chemoresistant Breast Cancer Cells and Can Enter Chemosensitive Breast Cancer Cells, Resulting in a Switch to Chemoresistance. We reasoned that TrpC5 might be released as cargo of EVs and further investigated the release and intercellular transfer of EVs by detecting TrpC5-containing, flotillin2-positive EVs in the culture medium of chemoresistant MCF-7/ADM cells (Fig. 2*A*). SEM (Fig. S4) and immuno-TEM (Fig. 2*B*) in freshly isolated EVs confirmed their identity with flotillin2. The acetomethoxy derivate of Calcein (Calcein-AM) staining confirmed their functional activity (Fig. S5 *A* and *B*). Because RNAs from EVs contain transcripts of tumor origin (4, 23), the presence of flotillin2 and MUC1 transcripts was first confirmed in isolated circulating

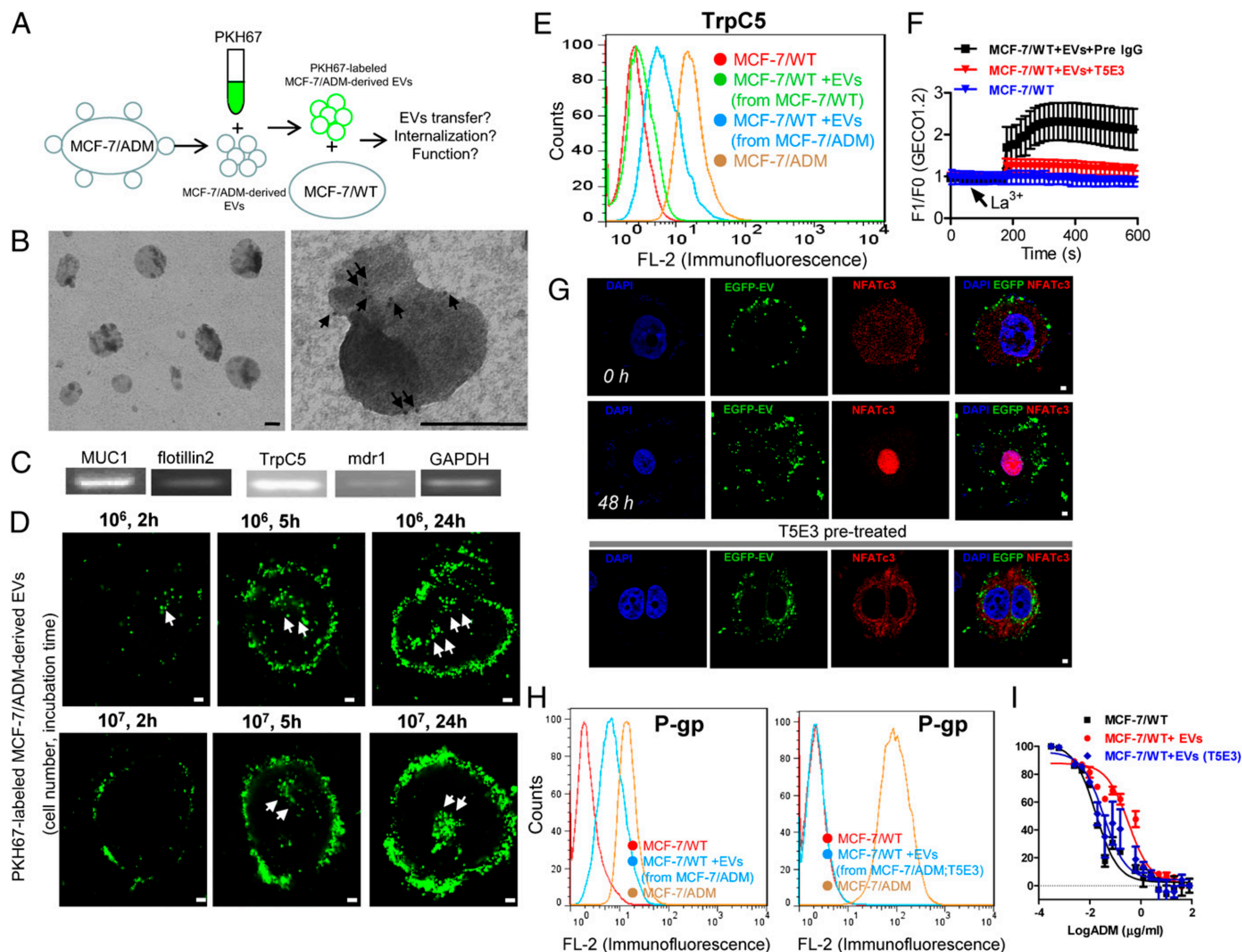

Fig. 2. TrpC5-containing circulating EVs are released from chemoresistant breast cancer cells and can enter chemosensitive breast cancer cells, resulting in a switch to chemoresistance. (*A*) Diagram depicting the experimental protocol. (*B*) Transmission electron micrograph (*Left*) and immunotransmission electron micrograph (*Right*) of flotillin2 expression in EVs isolated from culture medium of MCF-7/ADM cells. (Scale bars: 200 nm.) (*C*) RT-PCR assays showing the expression of four pooled transcripts (TrpC5, flotillin 2, *mdr1*, and MUC1) in EVs isolated from culture medium of MCF-7/ADM cells. (*D*) Confocal micrographs showing EVs labeled with PKH67 green fluorescence from MCF-7/ADM cells internalized into recipient MCF-7/WT cells in a time-dependent manner (cocultured for 2, 5, and 24 h) and dose-dependent manner (10^4 MCF-7/WT recipient cells cocultured with EVs from 10^6 or 10^7 MCF-7/ADM donor cells). Arrows indicate PKH67-positive signals/EVs inside the recipient cells. (Scale bars: 1 μm.) (*E*) FACS analysis showing a prominent shift in TrpC5 immunofluorescence of MCF-7/WT cells after 24 h of incubation with EVs from MCF-7/ADM cells. (*F*) $[Ca^{2+}]_i$ measurement using green intensiometric genetically encoded calcium indicators for optical imaging version 1.2 (GECO1.2) showing that the transferred TrpC5 is functional. Shown are time courses of $[Ca^{2+}]_i$ changes in response to La^{3+} in MCF-7/WT cells after 24 h of incubation with EVs from MCF-7/ADM cells (La^{3+}, 100 μM). (*G*) Confocal micrographs showing migration of NFATc3 from cytosol to nucleus after 48 h of incubation with GFP-labeled EVs from MCF-7/ADM cells. (Scale bars: 1 μm.) (*H*) FACS analysis showing a prominent shift in P-gp immunofluorescence of MCF-7/WT cells after 24 h of incubation with EVs from MCF-7/ADM cells. (*I*) The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays showing that coincubation with EVs from MCF-7/ADM cells increases the ADM resistance in recipient MCF-7/WT cells. If needed, EVs derived from MCF-7/ADM donor cells were pretreated with a TrpC5-specific blocking antibody (T5E3) or preimmune IgG at 20 μg/mL. Values are means ± SEMs.

EVs. MUC1 is frequently expressed in breast cancer; it is sorted into rafts by a flotillin-dependent mechanism and exported through EVs (24–26). Its expression in MCF-7/ADM cells was confirmed by immunostaining (Fig. S6). RT-PCR was then performed to simultaneously identify the transcript expression of flotillin2, MUC1, TrpC5, and *mdr1*. We found that these transcripts were positive in EVs derived from the culture medium of MCF-7/ADM cells, indicating the release of TrpC5-containing, flotillin2-positive EVs by these cells (Fig. 2*C*). To determine whether the EVs were internalized by recipient cells, we labeled the isolated EVs with a PKH67 labeling kit and incubated them with MCF-7/WT cells in culture (4, 27). The results showed that the labeled EVs bound to MCF-7/WT cells in a time- and dose-dependent manner (Fig. 2*D*). We further determined the transfer of EVs containing protein by monitoring GFP that was stably expressed in MCF-7/ADM cells. Uptake of GFP fluorescence into recipient cells was observed on exposure to the GFP-containing EVs (Fig. S7). In this regard, we asked whether TrpC5 can be transferred in this manner and remain functional. Fluorescence-activated cell sorting analysis showed that MCF-7/WT cells incubated with donor cell (MCF-7/ADM) EVs displayed an up-regulated expression of TrpC5, indicating the microvesicular transfer of TrpC5 (Fig. 2*E*). The functional presence of TrpC5 was determined by $[Ca^{2+}]_i$ measurement. Lanthanum (La^{3+}) is known to potentiate TrpC5 activity but inhibit many other Ca^{2+}-permeable channels (21, 22). Application of La^{3+} elicited a rise in $[Ca^{2+}]_i$ in recipient MCF-7/WT cells cocultured with EVs derived from MCF-7/ADM donor cells but not MCF-7/WT cells alone (Fig. 2*F*). This La^{3+}-elicited $[Ca^{2+}]_i$ rise was reduced by a TrpC5-specific blocking antibody, T5E3, confirming the involvement of functional TrpC5 (Fig. 2*F*). We showed previously that $[Ca^{2+}]_i$ influx through TrpC5 is crucial for nuclear translocation of activated T-cells isoform c3 (NFATc3) and that NFATc3 is the transcriptional factor that links the TrpC5 activity to P-gp production (21). Here, after 48 h of coculture with EVs, NFATc3 translocated into the nucleus in one-half of the cells (Fig. 2*G*), and P-gp increased in recipient MCF-7/WT cells (Fig. 2*H*). With EV transfer, the recipient chemosensitive MCF-7/WT cells became more resistant to ADM-induced cell death, displaying a 20-fold higher resistance to ADM (Fig. 2*I*). Importantly, NFATc3 translocation, P-gp increase, and up-regulation of drug resistance were markedly inhibited by pretreatment of EVs with T5E3 (Fig. 2 *G*–*I*), indicating that the up-regulated expression of P-gp was mainly caused by the transferred TrpC5-induced TrpC5–NFATc3–P-gp signal pathway (Fig. 2 *E*–*I*).

TrpC5 Is Required for the Release of Circulating EVs from Human Breast Tumor Xenografts in Athymic Nude Mice. After validating the generation and release of EVs from chemoresistant MCF-7/ADM cells, we next assessed circulating EVs in nude mice bearing MCF-7/ADM xenograft tumors. Immunohistochemistry showed that TrpC5, flotillin2, and P-gp were abundantly expressed in MCF-7/ADM xenograft tumors (Fig. 3*A* and Fig. S8) and that flotillin2 and P-gp expressions were substantially reduced in TrpC5–siRNA-treated tumor xenografts (Fig. 3*A*). RT-PCR was performed to determine the expression with four pooled transcripts TrpC5, flotillin2, *mdr1*, and MUC1 in circulating EVs. All transcripts were positive in seven of seven nude mice, indicating that TrpC5-containing EVs are released into the peripheral blood from MCF-7/ADM cells (Fig. 3*B*). Importantly, in the TrpC5–siRNA-treated tumor xenografts, the TrpC5, flotillin2, and P-gp transcript levels were lower than with scrambled siRNA, further showing the requirement of TrpC5 for the release of EVs and their cargos.

Circulating TrpC5-Containing EVs Predict Clinical Outcome of Chemotherapy for Breast Cancer. To explore the clinical potential of TrpC5 in the formation of EVs in breast cancer, we analyzed paired breast cancer tissue from 26 patients before and after anthracycline/taxane-based chemotherapy (Table S1). Immunohistochemistry showed that both TrpC5 and flotillin2 expressions were significantly up-regulated after chemotherapy (Fig. 4 *A* and *B*). Moreover, TrpC5 expression was positively correlated with that of flotillin2 (Fig. 4*C*). Treatment response was assessed by the response evaluation criteria in solid tumors (28). Among 26 patients, 13 patients responded to chemotherapy [partial response/complete response (PR/CR)], whereas 13 patients were not responsive [progressive disease/stable disease

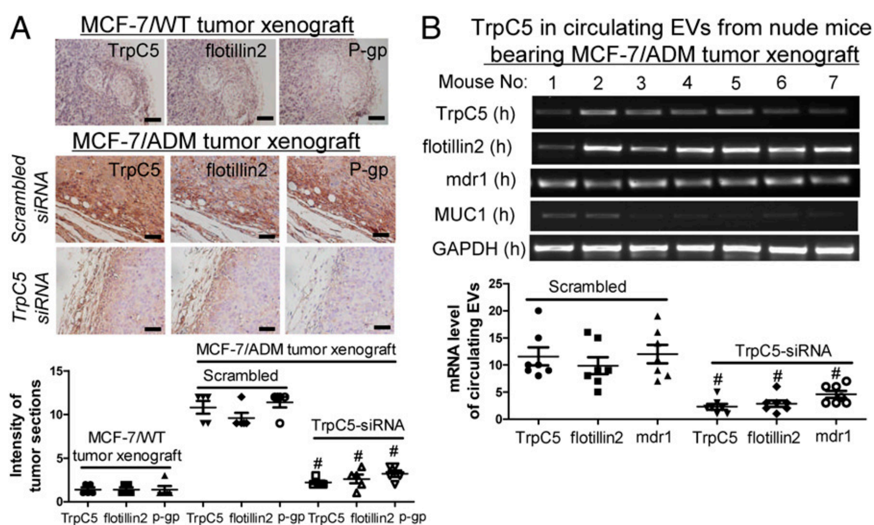

Fig. 3. TrpC5 is required for the release of circulating EVs from human breast tumor xenografts in athymic nude mice. (*A*) Flotillin2 expression was substantially reduced in TrpC5–siRNA-treated tumor xenografts. Immunohistochemical staining for TrpC5, flotillin2, and P-gp in sections of MCF-7/ADM tumor xenografts with TrpC5-siRNA (scrambled siRNA as control). Immunohistochemical staining in sections of MCF-7/WT tumor xenografts was taken as the control ($n = 5$ in each group). (*B*) RT-PCR assays showing the expression of four pooled transcripts (TrpC5, flotillin 2, *mdr1*, and MUC1) in peripheral blood from nude mice bearing chemoresistant MCF-7/ADM tumor xenografts. Female nude mice bearing xenograft tumors derived from MCF-7/ADM were injected at the tumor sites with TrpC5-siRNA (40 pmol; scrambled siRNA as control; $n = 7$ in each group). MCF-7/ADM tumor continued to grow in size under ADM treatment, indicating ADM resistance. Data were analyzed by Student *t* test. Values are means ± SEMs. $^{\#}P < 0.05$ compared with scrambled. (Scale bars: 100 μm.)

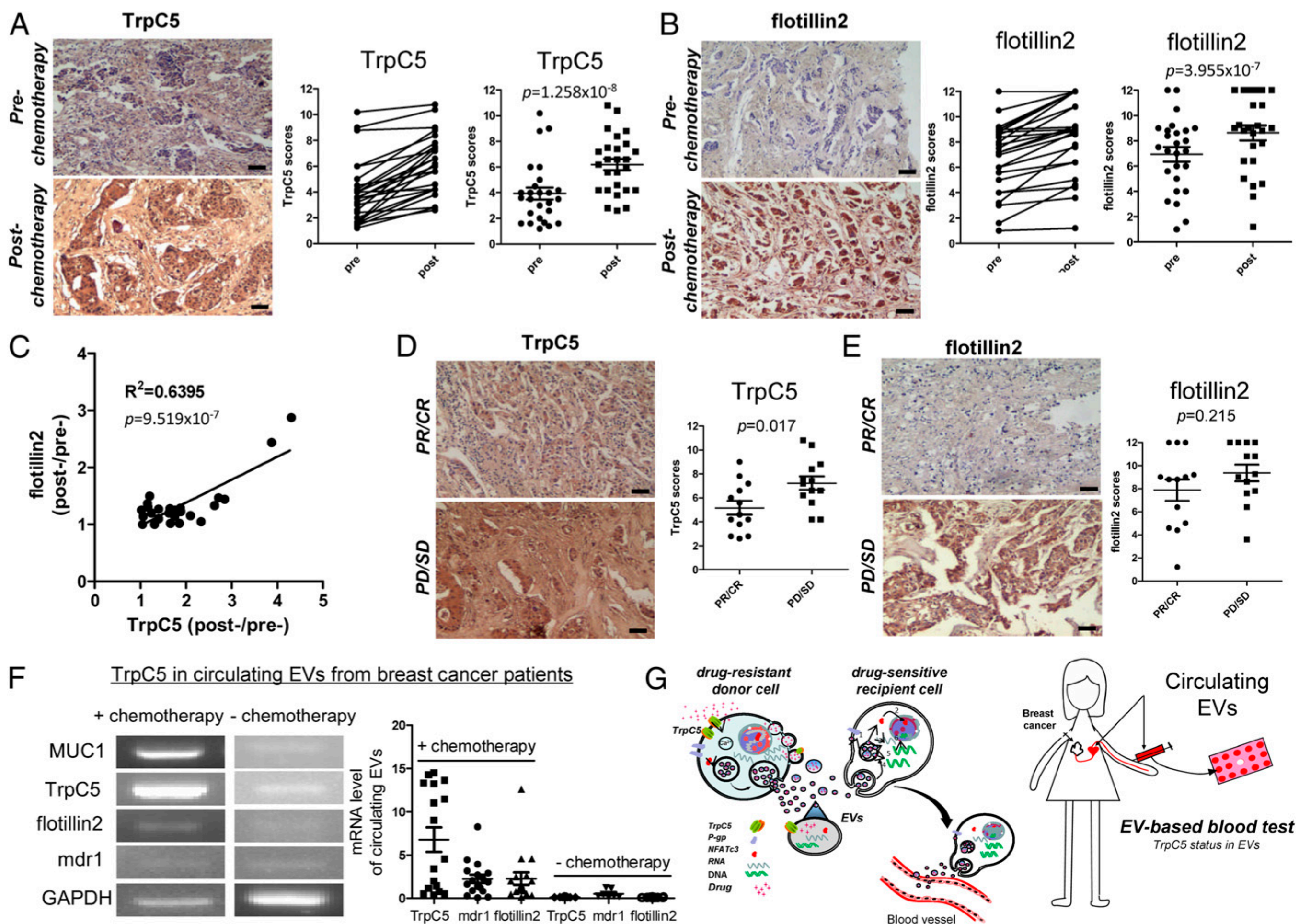

Fig. 4. Circulating TrpC5-containing EVs predict clinical outcome of breast cancer with chemotherapy. (*A*) Representative images and summary data from immunohistochemical staining of TrpC5 in paired pre- and postchemotherapy breast cancer tissue from patients showing elevated TrpC5 expression (n = 26). (*B*) Representative images and summary data from immunohistochemical staining for flotillin2 in paired pre- and postchemotherapy breast cancer tissue showing that flotillin2 expression is elevated in breast cancer patients (n = 26). Data were analyzed by Student t test. (*C*) Pearson correction of TrpC5 expression with flotillin2 (n = 26). Data were analyzed using Pearson correlation test. (*D*) TrpC5 expression was significantly greater in patients with PD/SD (n = 13) compared with patients with PR/CR (n = 13). (*E*) Flotillin2 expression was higher in patients with PD/SD (n = 13) than in patients with PR/CR (n = 13). Data were analyzed by Student t test. (*F*) RT-PCR assays showing the expression of four pooled transcripts (TrpC5, flotillin 2, *mdr1*, and MUC1) in peripheral blood were elevated in patients who received chemotherapy with PD/SD (n = 17) but not elevated in patients who did not received chemotherapy (n = 12). (*G*) Proposed model highlighting TrpC5 in EVs formation and transfer and the diagnostic implications. (Scale bars: 100 μm.)

(PD/SD)]. Importantly, TrpC5 expression was significantly greater in patients with PD/SD than in patients with PR/CR (Fig. 4*D*), indicating its close association with the induction of chemoresistance. Flotillin2 expression was also higher in patients with PD/SD than patients with PR/CR (Fig. 4*E*). RT-PCR was next used to assess the features of TrpC5-containing circulating EVs in peripheral blood from 33 patients with chemotherapy. TrpC5 as well as flotillin2, *mdr1*, and MUC1 were simultaneously amplified from EVs in 17 of 33 samples from these patients but not in 12 patients without chemotherapy (Fig. 4*F* and Table S2). Flotillin2 and TrpC5 expression or MUC1 and TrpC5 expression in circulating EVs isolated from patients was further confirmed by FACS (Fig. S9). Calcein-AM staining in circulating EVs confirmed their functional activity (Fig. S5*B*). Thus, identification of tumor-specific TrpC5 in circulating EVs may provide a window on the clinical outcome of chemotherapy (Fig. 4*G*).

Discussion

To achieve more effective and individualized chemotherapeutic treatment of breast cancer patients, it is essential to understand the mechanisms responsible for drug resistance and define reliable indicators for response to therapy. The findings described here shed light on an unconventional and poorly understood mechanism of cell-to-cell communication in the context of chemotherapeutic drugs and how that communication may have significant consequences in the development and transfer of the chemoresistant phenotype in breast cancer. In particular, we have shown that exposing chemosensitive breast cancer recipient cells to bioactive TrpC5-containing EVs that are constitutively shed by certain chemoresistant breast cancer donor cells can cause the recipient cells to acquire a chemoresistant phenotype. To the best of our knowledge, this is the first study investigating the association of an ion channel in circulating EVs with chemotherapeutic response.

It has been suggested that EVs can transfer P-gp from drug-resistant lymphoblastic leukemia cells to nonresistant cells, conferring multidrug resistance on nonresistant cells, but its capacity is not sustained (29). We recently reported that up-regulation of TrpC5 protein is crucial for P-gp induction and the development of chemoresistance in breast cancer cells (21). Here, we show that the same is true for recipient cells after their EV-mediated acquisition of resistance. Transfer of TrpC5 allowed

the recipient cells to acquire/produce this Ca^{2+}-permeable channel, consequently stimulating P-gp production in the recipient cells through a Ca^{2+}- and NFATc3-mediated mechanism and as a result, endowing nonresistant cells with chemoresistance. Ca^{2+} entry is also known to stimulate the transcription of TrpCs themselves in a positive feedback manner through the NFAT transcription factor (30, 31). In this way, TrpC5 and P-gp can be continuously produced, resulting in long-lasting drug resistance.

One aspect of our work that merits additional consideration involves the potential mechanisms of abundant accumulation of TrpC5 in EVs in the context of chemotherapeutic drugs. A growing body of evidence has confirmed the translocation of Trp channels. A particularly good example comes from a recent study, which showed that EGF induces rapid vesicular translocation of TrpC5 channels, dramatically increasing membrane-associated functional TrpC5 and resulting in tight spatial–temporal control of TrpC5 (15). Rho-GTPases regulate vesicular trafficking and membrane processes, and Rac1 plays a role in the translocation of functional TrpC5 (15). Thus, more work needs to be done to determine the molecular mechanism of EV accumulation of TrpC5 in chemoresistant breast cancer cells.

Collectively, our data suggest that up-regulated TrpC5 accumulated in EVs is responsible for EV formation and EV trapping of chemotherapeutic drugs. More importantly, we also showed a critical role of TrpC5-containing EVs in the transfer of drug resistance property to nonchemoresistant recipient cells. Notably, we found a striking association between TrpC5-containing EVs circulating in peripheral blood and the clinical response to chemotherapy. Although there is no reason to think that these effects are limited to TrpC5, TrpC5-containing circulating EVs may enable the monitoring of chemotherapeutic efficacy. Thus, it may be worthwhile to further explore the potential of using TrpC5-containing EVs as a diagnostic biomarker for chemoresistant breast cancer.

Materials and Methods

Additional methods are described in *SI Materials and Methods*.

TEM. Sample preparation, thin sectioning, and immunolabeling on sections were performed with antibodies against ADM, TrpC5, and flotillin2 and gold-coupled secondary antibodies. Sections were viewed with a Hitachi HT7700 TEM.

ADM Accumulation. Subcellular distribution of ADM was determined using a laser scanning confocal microscope.

Labeling of EVs with PKH67 and Confocal Microscopy. EVs were labeled with a PKH67 green fluorescent labeling kit (Sigma-Aldrich) following the manufacturer's instructions. MCF-7/WT cells were incubated with the PKH67-labeled MCF-7/ADM-derived EVs for the indicated time. Cells were washed and subjected to confocal microscopy (Zeiss LSM 510 confocal laser scanning microscopy).

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