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Tumor endothelial expression of P-glycoprotein upon microvesicular transfer of TrpC5 derived from adriamycin-resistant breast cancer cells

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ABSTRACT

Treatment of carcinoma commonly fails due to chemoresistance. Studies have shown that endothelial cells acquire resistance *via* the tumor microenvironment. Microvesicle (MV) shedding from the cell membrane to the microenvironment plays an important role in communication between cells. The aim of the present study was to determine whether MCF-7 adriamycin-resistant cells (MCF-7/ADM) shed MVs that alter the characteristics of human microvessel endothelial cells (HMECs). MVs from tumor cells transferred a Ca^{2+}-permeable channel TrpC5 to HMECs, inducing the expression of P-glycoprotein (P-gp) by activation of the transcription factor NFATc3 (nuclear factor of activated T cells isoform c3). Expression of the *mdr1* gene was blocked by the TrpC5-blocking antibody T5E3, and the production of P-gp in HMECs was reduced by blockade of TrpC5. Thus, we postulate that endothelial cells acquire the resistant protein upon exposure to TrpC5-containg MVs in the microenvironment, and express P-gp in the TrpC5–NFATc3 signal pathway.

1. Introduction

Since Folkman proposed the idea that tumor growth relies on angiogenesis [1], tumor blood vessels have become a potential target for cancer therapy. Unfortunately, clinical trials have shown that some patients do not benefit from this treatment [2]. The mechanisms involved in the resistance to anti-angiogenic therapy are complicated. When vascular endothelial growth factor (VEGF) is blocked, other pro-angiogenic factors are expressed as compensation to prevent anti-angiogenic therapy [3,4]. Another mechanism is that stimulating the expression of cytokines in non-tumor tissues acts against anti-angiogenesis and promotes metastasis in a VEGF-independent manner [5]. Hypoxia also plays important roles in the resistance of endothelial cells (ECs) [6]. The emergence of resistance in tumor blood vessels also contributes to anti-angiogenic failure [7]. Recent studies have reported that the tumor microenvironment greatly influences the drug-resistance of ECs [8–10], but the mechanism by which ECs acquire resistance from their surroundings is poorly understood.

The microenvironment influences tumor cells through the shedding of membrane microvesicles (MVs) [11–13]. MVs are classified as exosomes, microvesicles, and micro-particles by their nature, size, and origin [14]. As cargos, MVs contain much information, nucleic acids, specific proteins, and micro-RNAs [15,16]. These characteristics of MVs indicate that they play a vital role in tumor progression [17,18]. Microvesicles and exosomes readily interact with ECs [19,20], implying that MVs are involved in the plasticity of the characteristics of ECs. The role of MVs in the acquisition of resistance by ECs is not clear.

Here, we focused on how MVs derived from MCF-7 adriamycin-resistant cells (MCF-7/ADM) communicate with and influence the resistance of human microvessel endothelial cells (HMECs). We had already demonstrated that P-gp expression is modulated by the transient receptor potential channel TrpC5 in the transcription factor NFATc3 signal pathway in MCF-7 adriamycin-resistant cells [21]. Our present study demonstrated that MCF-7/ADM cell-derived MVs transferred both P-gp and TrpC5 to HMECs, and TrpC5-containing MVs modulated the expression of P-gp in HMECs *via* the translocation of the transcription factor NFATc3.

2. Materials and methods

2.1. Cells and reagents

Both MCF-7 wild-type (MCF-7/WT) and Human microvessel cells (HMECs) were obtained from the ATCC, and were cultured

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in DMEM and RPMI1640, respectively, with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin. Adriamycin-resistant human breast cancer cells (MCF-7/ADM) were cultured with RIPM1640 and 10% FBS, and all breast cancer cells were cultured with 0.01 mg/mL human recombinant insulin. All cells were incubated at 37 °C in 5% CO_2 humidified air.

The PKH67 Fluorescent Cell Linker kit was from Sigma (St Louis, MO, USA); MTS was from Promega (Madison, WI, USA); NFATc3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); TrpC5 and P-gp antibodies were from Abcam (Cambridge, UK); actin antibody was from Biotime (Shanghai, China); human P-gp antibody and mouse CD31 antibody for tumor analysis were obtained from Santa Cruz Biotechnology.

2.2. Adriamycin and paclitaxel chemosensitivity assay

The cells were plated at 6000 per well (in triplicate) in 96-well plates. After 24 h, they were treated with adriamycin or paclitaxel for 48 h. After the treatment, MTS was added to the wells and incubated at 37 °C with 5% CO_2 for 4 h. Absorbance at 590 nm was measured with a microplate reader (Thermo, Synergie HT, USA).

2.3. Electron microscopy

To visualize MVs, pellets were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature for 1 h. After washing with PBS, the fixed pellets were postfixed in 1% OsO_4 for 2 h at 4 °C, dehydrated in graded ethanol (v/v, 30%, 50%, 70%, and 100%) and a 1:1 mixture of 90% ethanol and 90% acetone, and washed 3 times in acetone. Next, the cells were infiltrated with a graded series of Spurr resin in absolute acetone (25%, 50%, and 75%) for 45 min at each step. Finally, the cells were placed in 100% spur resin and kept overnight at 4 °C. The samples were placed in BEEM capsules at room temperature for 4 h, and the capsules were embedded for 2 days at 60 °C. For observation, ultrathin sections (70 nm) were cut with a diamond knife and collected on 200-mesh coated copper grids. After contrasting with uranyl acetate and lead citrate, the grids were examined on a transmission electron microscope (Hitachi H7700 TEM, Japan).

2.4. Adriamycin accumulation assay

The distribution of adriamycin in MCF-7/WT and MCF-7/ADM cells was determined using a laser scanning confocal microscope (Leica TCS SP8, Germany) with a ×63 oil-immersion objective lens. The cells were treated with adriamycin overnight and fixed in 4% paraformaldehyde, then stained with DAPI, and assessed under a confocal microscope. Adriamycin fluorescence was determined at an excitation wavelength of 488 nm and the emission was collected at 560 nm.

2.5. Immunofluorescence staining

HMECs were plated at 1×10^4 cells/mL, and serum-starved (RIPA 1640, 2% FBS) for 24 h. Then cells were incubated with or without MVs for 24 h. Cells were fixed in 4% paraformaldehyde after overnight incubation with MVs, and permeabilized with 0.1% Triton-X 100 for 10 min at room temperature. The samples were blocked with 2% BSA in PBS for 30 min at room temperature, incubated with primary antibody against NFATc3 overnight at 4 °C, and then incubated with fluorescent secondary antibody for 1 h at room temperature. Then images were acquired by a confocal laser scanning microscope with a ×63 oil-immersion objective lens.

2.6. Collection of microvesicles

MVs were prepared by stepwise centrifugation. A low speed removed dead cells and large particles of cell debris. Then ultracentrifugation for 1 h obtained MVs. Then MVs pre-stained with PKH67 were co-cultured with HMECs for the indicated times, and then observed using a laser scanning microscope.

2.7. Western-blot

Protein was obtained from cells and was lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and PMSF protease inhibitor. Equal protein loading of the lysates was achieved by standardization with the BCA protein assay kit from Biotime (Shanghai, China). Samples were separated on SDS–PAGE gels and transferred to nitrocellulose transfer membranes. After blocking with 5% skim milk in TBST, the membranes were incubated with the appropriate primary antibody (actin, P-gp, or TrpC5) overnight at 4 °C, followed by 2 h of incubation with HRP-conjugated secondary antibodies. The protein bands were visualized with ECL kits.

2.8. Immunofluorescence by flow cytometry

The cells were plated in 6-well plates for 12 h. HMECs were co-cultured for 5 h with MVs derived from MCF-7/ADM cells. Then, HMECs and cells exposed to MVs were harvested using trypsin digestion with 2 mM EDTA and washed with PBS. Cells were fixed in 4% paraformaldehyde and stained with primary antibody against P-gp or TrpC5 for 1 h. Then cells were incubated at room temperature with fluorescent secondary antibodies. Finally, samples were re-suspended in PBS and analyzed using a FACScalibur flow cytometer (BD Biosciences, USA).

2.9. Reverse transcriptase (RT)-PCR

Total RNA was extracted using the Trizol protocol and cDNA was synthesized from the mRNA using a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, USA) for RT-PCR. PCR was performed with 30 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s. The data were analyzed with ImageJ software (NIH, Bethesda, MD, USA). GAPDH was used as endogenous control. The primer sequences were: GAPDH: 5′-tggagtccactggcgtcttc-3′ and 5′-gcttgacaaagtggtcgttgag-3′; P-gp: 5′-ttggcagggaaagtgctgcttgat-3′ and 5′-ttgttccagcctggacactgaccat-3′.

2.10. Tumor analysis

MCF-7/ADM cells were injected into the flanks of female nude mice (5×10^6 cells per mouse) and allowed to propagate for 4–8 weeks. All procedures were performed according to the guidelines of the University of Jiangnan Animal Care and Use Committee. Tumor tissues were harvested, and fixed in 4% paraformaldehyde, sectioned (5 μm), and stained for human P-gp and mouse CD31 antibodies, and incubated with the respective secondary antibodies tagged with Alexa Fluor Cy3 or Alexa Fluor 488. Images were collected using the confocal microscope.

2.11. Statistics

All experiments were reproduced at least twice with similar results. Quantitative data are presented as the average value of replicates within the representative experiment ± SD (standard deviation).

3. Results

3.1. Characteristics of microvesicle shedding from MCF-7 adriamycin-resistant cells

Adriamycin is commonly used as a powerful chemotherapeutic drug in breast cancer treatment [22]. It mainly intercalates DNA and induces the death of cells. MCF-7/ADM cells were obtained by stepwise induction with adriamycin, and displayed about a 600-fold greater resistance to adriamycin than the parental wild-type MCF-7 line (MCF-7/WT) (Fig. 1A). MCF-7/ADM cells also displayed resistance to paclitaxel compared to MCF-7/WT cells (Fig. 1A). Adriamycin was selected to investigate the microvesicular trapping of chemotherapeutic drugs because of its natural red fluorescence [23]. The subcellular distribution of adriamycin was consistent with other reports. The wild-type cells accumulated adriamycin in the nucleus, but in MCF-7/ADM cells the accumulation was almost entirely in the cytoplasm where it was trapped in microvesicles (Fig. 1B). Moreover, the surface of MCF-7 adriamycin-resistant cells was covered with microvesicles varying in size from 50 to 500 nm (Fig. 1C). Also we found greatly increased generation of microvesicles on the surface of MCF-7/ADM cells, compared to the wild-type (Fig. 1C).

The characteristics of MVs derived from MCF-7/ADM cells were further investigated. P-glycoprotein (P-gp) is a classical drug-resistance protein encoded by the *mdr1* gene, and it pumps chemotherapeutic drugs out of cells [24]. TrpC5 is a member of TRP channel family, and plays vital roles in diverse physiological processes [25]. Excessive expression of TrpC5 channels is associated with multi-drug resistance. We assessed the expression of P-gp and TrpC5 by confocal laser scanning microscopy. The protein expression was increased in MCF-7/ADM cells, but faintly detectable in MCF-7/WT cells (Fig. 1D). Confocal images showed P-gp and TrpC5 as spots on the surface of MCF-7/ADM cells. This implied that P-gp and TrpC5 might be contained in MVs derived from MCF-7/ADM cells. This result was confirmed by Western-blot analysis of MVs derived from MCF-7/ADM and MCF-7/WT cells (Fig. 1E). Furthermore, MVs were positive for flotillin-2 [12]. These results implied that the transfer of drug-resistant characteristics to ECs mainly depends on drug-resistant tumor cells.

3.2. Uptake of P-gp and TrpC5 by HMECs on exposure to MVs containing P-gp and TrpC5 derived from MCF-7/ADM cells

We found that neither P-gp nor TrpC5 was detectable in HMECs, as well as in MVs as assessed by Western blot (Fig. 2A). The collected MVs were positive for flotillin-2 (Fig. 2A). This huge difference between MCF-7/ADM cells and HMECs, strongly suggested that drug-resistant cells might change the resistance of normal ECs. The influence of MCF-7/ADM cells on HMECs might be due to MVs containing these drug-resistant proteins.

PKH67, a membrane stain, was used to monitor the intercellular transfer of MVs [17]. MVs collected from 10^7 MCF-7/ADM donor cells were labeled with PKH67. Then the labeled MVs were incubated with HMECs for 2, 5, or 24 h. Images token by confocal microscopy showed that MVs were taken into HMECs in a time-dependent manner (Fig. 2B). It was notable, after incubation with MVs, that the morphology of HMECs was changed dramatically, from flat, elongated, and spindle-like to various shapes.

Fluorescence-activated cell sorting (FACS) was further used to detect the transfer of protein-containing MVs [26]. HMECs displayed prominent shifts in P-gp and TrpC5 immunofluorescence after incubation with MVs (Fig. 2C). This clearly demonstrated that MCF-7/ADM cells shed MVs, and transferred P-gp and TrpC5 to HMECs. TrpC5 is known to modulate P-gp expression in MCF-7/ADM cells, so we investigated whether this also happened in co-cultured HMECs.

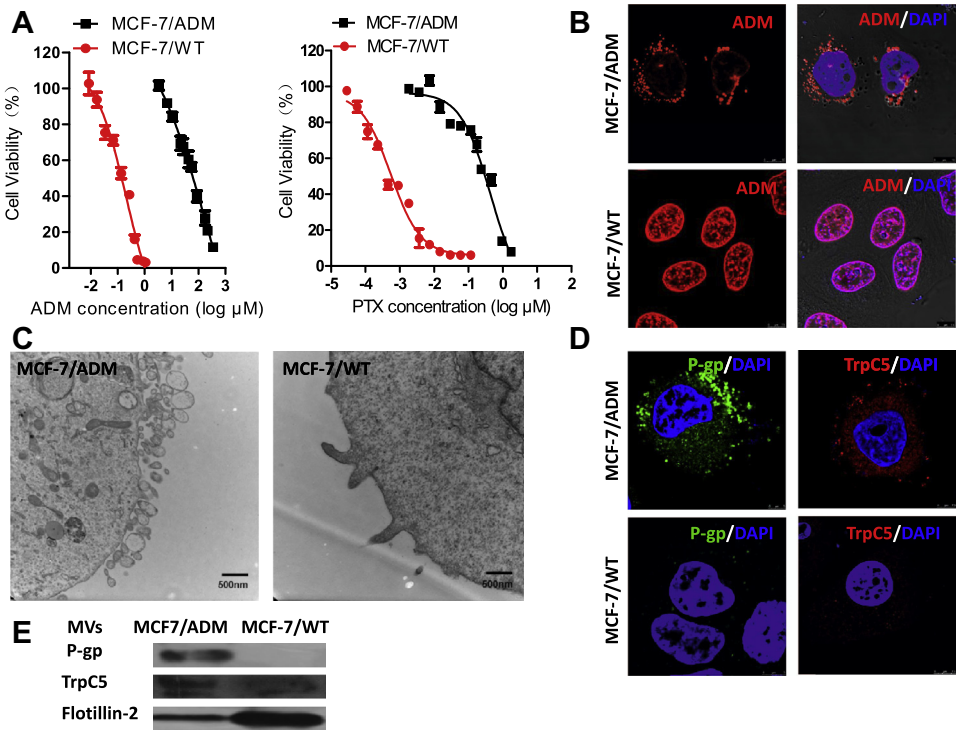

Fig. 1. Characteristics of MCF-7 adriamycin-resistant cells (MCF-7/ADM) and their MVs. (A) Sensitivity of MCF-7/ADM and wild-type cells (MCF-7/WT) to adriamycin and paclitaxel. (B) Subcellular distribution of adriamycin in MCF-7/ADM and MCF-7/WT cells. Images were acquired by confocal microscopy. (C) Transmission electron microscopic images of MCF-7/ADM and MCF-7/WT cells. (D) Expression of P-gp and TrpC5 in MCF-7/ADM and MCF-7/WT cells detected by confocal laser scanning microscopy. P-gp and TrpC5 were mainly distributed in microvesicles from MCF-7/ADM cells. (E) Expression levels of P-gp and TrpC5 in MVs from MCF-7/WT and MCF-7/ADM cells detected by Western blot. MVs were positive for the microvesicle marker, flotillin-2.

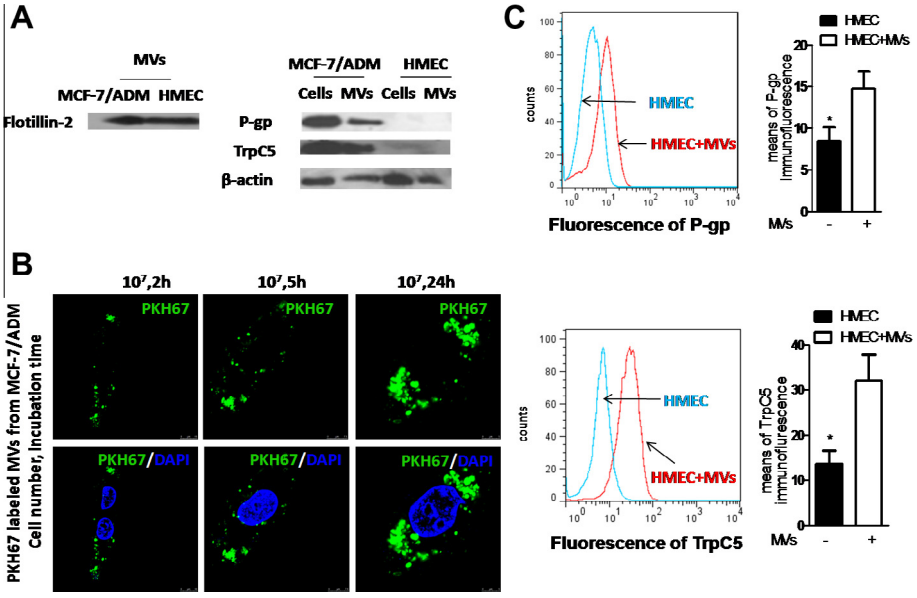

Fig. 2. Uptake of P-gp and TrpC5 by HMECs on exposure to MVs from MCF-7/ADM cells. (A) Expression levels of P-gp and TrpC5 on MCF-7/ADM cells, HMECs, and associated MVs detected by Western blot. MVs were positive for the microvesicle marker flotillin-2. Protein loading was quantified by β-actin. (B) MVs from MCF-7/ADM cells were adherent on HMECs in a time-dependent manner. (C) Uptake of P-gp and TrpC5 by HMECs on exposure to MVs from MCF-7/ADM cells. The immunofluorescences of P-gp and TrpC5 showed significant shifts after co-culture with MVs, $^{*}p < 0.05$ compared to HMECs.

3.3. MVs promote the signal pathway of P-gp expression by translocation of the transcription factor NFATc3

The Ca^{2+}-dependent transcription factor NFATc3 [27,28], *via* the TrpC5–NFATc3 pathway, stimulates the transcriptional activity of the *mdr1* promoter in breast cancer cells [21]. So, we set out to determine whether P-gp is expressed in HMECs upon exposure to TrpC5-containing MVs *via* the same signaling pathway.

We noted that NFATc3 was partially or fully translocated from the cytoplasm to the nucleus when incubated with MVs derived from MCF-7/ADM cells (Fig. 3A). This indicated that NFATc3 was activated when MVs transferred TrpC5 to HMECs. To determine whether TrpC5 regulated P-gp production, we blocked the activity of TrpC5 with the specific blocking antibody T5E3 [29] in MVs from MCF-7/ADM cells, then measured the immunofluorescence of P-gp. We found that when MVs were pretreated with T5E3, the P-gp fluorescence of HMECs diminished, compared to those without T5E3 (Fig. 3B). Furthermore, we found that the level of P-gp mRNA was decreased in HMECs when MVs were pretreated with T5E3 (Fig. 3C). These results strongly implied that TrpC5-derived MVs not only carry P-gp to HMECs, but also stimulate the expression of P-gp *via* the TrpC5–NFATC3 signal pathway.

3.4. Transfer of tumor-derived P-gp to endothelial cells in vivo

To confirm the role of MVs in communication between drug-resistant cells and ECs *in vivo*, MCF-7/ADM cells were injected into the flanks of female nude mice (BALB/cAnNCr-nu/nu). Immunostaining of tumor tissue showed that the fluorescent signals of the mouse EC marker CD31 [30] and human P-gp staining were merged at the same sites (Fig. 4A). Moreover, a much higher level of P-gp production was found in the vascular ECs of adriamycin-resistant tumor xenografts than in other sites. This investigation demonstrated that MCF-7/ADM cells shed MVs as cargos containing the resistant protein P-gp, and the cargo transferred information to ECs *in vitro* and *in vivo*. Inhibiting the activity of TrpC5 in MCF-7/ADM cells reduced the expression of P-gp in the xenografts, as well as its accumulation on tumor blood vessels (Fig. 4B). The level of P-gp located on blood vessels in tumors harvested from control cells was higher than in TrpC5-siRNA cells. This agrees with the finding that TrpC5 modulates P-gp production in ECs. We propose that a mechanism involved in the emergence of drug-resistance in tumor ECs is *via* TrpC5-containing MVs that transfer P-gp and induce endothelial expression of P-gp through the TrpC5-NFATc3 signaling pathway (Fig. 4C).

4. Discussion

In the present study, we demonstrated that MCF-7/ADM cells not only produced P-gp and TrpC5 but also released these proteins into the microenvironment in the form of MVs. The MVs transferred TrpC5 to ECs, and induced more P-gp expression *via* NFATc3 translocation.

Drug-resistance in ECs promotes tumor growth as well as metastasis [5], but the mechanisms by which ECs resistance is acquired are still poorly understood. We first demonstrated that ECs acquired resistant characteristics *via* MVs derived from drug-resistant cells. MVs readily interact with and trigger signal pathways in ECs. It is known that tumor-derived MVs transfer epidermal growth factor receptor to ECs, and stimulate the endothelial expression of VEGF [26]. Ovarian carcinoma cells express CD147 in MVs and stimulate pro-angiogenic activity in HUVECs in a CD147-dependent fashion [19]. We found that TrpC5-containing MVs from drug-resistant cells triggered signaling pathways in ECs.

Our previous study showed that TrpC5, a Ca^{2+} channel, regulates the expression of P-gp in drug-resistant cancer cells [21]. TrpC5 increases the intracellular Ca^{2+} level. Increasing $[Ca^{2+}]_i$ enhances P-gp expression in cancer cells [27,31]. Nuclear factor of activated T-cells is a Ca^{2+}-dependent transcription factor and readily stimulates the gene transcription through several TRP channels, including TrpC1 [32] and TrpC3 [33]. We reasoned that, after ECs were incubated with TrpC5-containing MVs, NFATc3 was stimulated by Ca^{2+} entry through TrpC5, and activated transcription activity to transcribe *mdr1* mRNA. Inhibiting the activity of TrpC5 reduced the level of P-gp in ECs both *in vitro* and *in vivo*, which is consistent with these investigations.

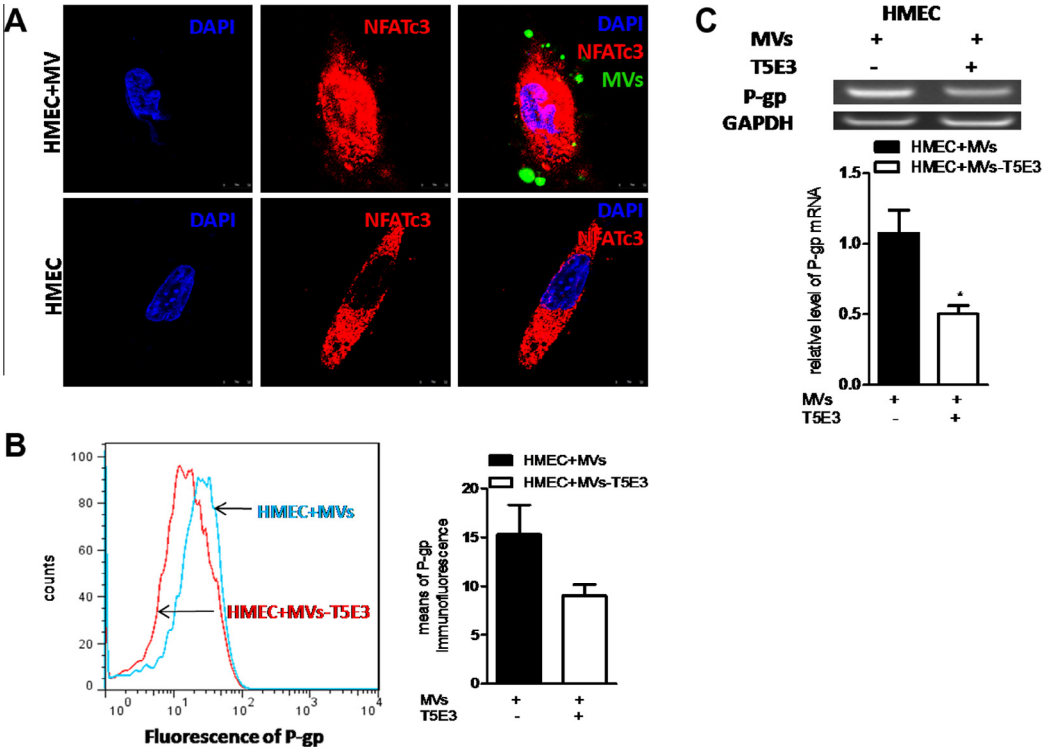

Fig. 3. MVs promoted the signal pathway of P-gp expression by translocation of the transcriptional factor NFATc3. (A) NFATc3 translocated upon the uptake of TrpC5-derived MVs by ECs. Serum-starved HMECs were incubated with MVs shed from MCF-7/ADM cells, and NAFTc3 translocated into the nucleus in some of the HMECs. (B) T5E3 blockade of TrpC5 activity down-regulated P-gp expression in HMECs. HMECs were incubated with T5E3-pretreated or untreated MVs for 24 h. After co-culture with MVs, HMECs were harvested and extensively washed in PBS. P-gp immunofluorescence was detected by flow cytometry. (C) The level of P-gp mRNA was decreased when TrpC5 was inhibited. The level of *mdr1* mRNA declined after incubation with T5E3-pretreated MVs, $^{*}p < 0.05$ compared to HMECs co-cultured with MVs.

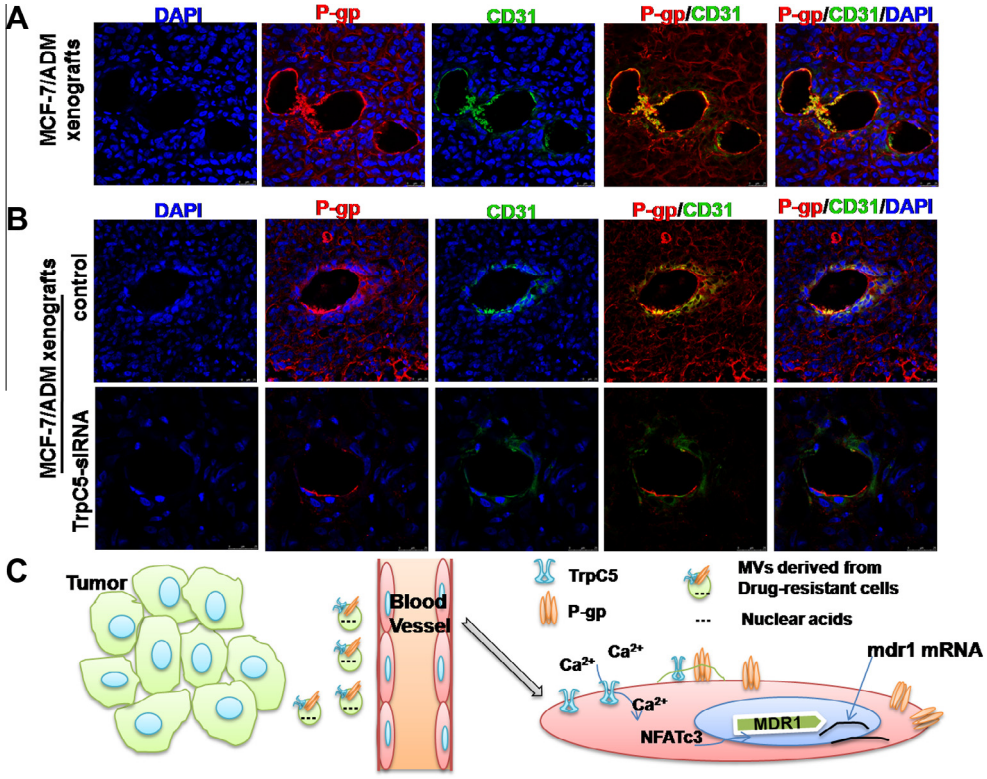

Fig. 4. Transfer of tumor-derived P-gp to endothelial cells *in vivo*. (A) Co-localization of immunofluorescence of human P-gp (red) and staining for the mouse endothelial marker CD31 (green) in MCF-7/ADM xenografts (nude mice). (B) Levels of P-gp located on blood vessels in tumor harvested from control and TrpC5-siRNA cells. (C) Proposed mechanism involved in the emergence of drug-resistance in tumor ECs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In vivo, the reasons for the lower expression of P-gp on ECs in TrpC5-siRNA xenografts are complicated. Reducing the level of TrpC5 disturbs the Ca^{2+} homeostasis in tumor cells. This not only down-regulates the expression of P-gp but also might suppress the formation of MVs. It has been reported that the $[Ca^{2+}]_i$ modulates the formation of MVs [34]. One mechanism for MVs formation is that a rise in $[Ca^{2+}]_i$ alters the asymmetric phospholipid distribution in the plasma membrane [35], and thus promotes the formation of MVs. Another mechanism is that Ca^{2+} also contributes to reorganization of the cytoskeleton and modulates MV formation by the activation of cytosolic proteases, such as calpain [14,34,35]. Blockade of TrpC5 activity reversed resistance in ECs and tumors. We propose that the blockade of TrpC5 might be a novel potential drug target to overcome drug-resistance in cancer.

In conclusion, our study demonstrated a novel mechanism of EC resistance acquisition by MVs shed from drug-resistant cells. Specifically, TrpC5-containing MVs induced expression of the resistant protein P-gp in ECs. Blockade of the activity of TrpC5 reversed the resistance, and this might be a promising drug target for cancer-resistance treatment.

Acknowledgments

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