Cancer Science
Japanese Cancer Association JCA
Open Access

Microparticles induce multifactorial resistance through oncogenic pathways independently of cancer cell type

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Key words
Inhibitors of apoptosis proteins, microparticles, multidrug resistance, NFκB, P-glycoprotein

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Funding Information
This study was supported by Instituto Nacional de Ciência e Tecnologia (INCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Oncobiologia and Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ).

Received August 8, 2014; Revised October 24, 2014; Accepted October 27, 2014

Cancer Sci **106** (2015) 60–68

doi: 10.1111/cas.12566

Multidrug resistance (MDR) is considered a multifactorial event that favors cancer cells becoming resistant to several chemotherapeutic agents. Numerous mechanisms contribute to MDR, such as P-glycoprotein (Pgp/ABCB1) activity that promotes drug efflux, overexpression of inhibitors of apoptosis proteins (IAP) that contribute to evasion of apoptosis, and oncogenic pathway activation that favors cancer cell survival. MDR molecules have been identified in membrane microparticles (MP) and can be transferred to sensitive cancer cells. By co-culturing MP derived from MDR-positive cells with recipient cells, we showed that sensitive cells accumulated Pgp, IAP proteins and mRNA. In addition, MP promoted microRNA transfer and NFκB and Yb-1 activation. Therefore, our results indicate that MP can induce a multifactorial phenotype in sensitive cancer cells.

Multidrug resistance (MDR) is a major chemotherapeutic obstacle in treating many neoplasms. The main mechanism of MDR is the overexpression of P-glycoprotein (Pgp/ABCB1), a 170-kDa transmembrane transporter protein that effluxes chemical compounds, including anticancer drugs, across the cell membrane.[1,2] Many studies have already demonstrated that Pgp is expressed in leukemias and solid tumors and that its overexpression is correlated to chemotherapy response.[3–5] Likewise, molecular mechanisms that are related to cell death pathway inhibition are involved in cancer resistance. The inhibitors of apoptosis proteins (IAP) family is deregulated in several cancers, and the survivin/BIRC5, XIAP/BIRC4 and cIAP1/BIRC2 members have been associated with an unfavorable cancer prognosis.[6]

The association of multiple mechanisms that contribute to the MDR phenotype in cancer cells has been previously discussed.[1,7] Recently, our group demonstrated that the induced co-expression of Pgp and survivin contributes to the MDR phenotype in leukemia cells.[8] In another study, we established a significant correlation between survivin and Pgp in chronic myeloid leukemia (CML) patients.[9] Similarly, we have also shown that XIAP protein expression significantly correlates to Pgp expression in CML patient samples, independent of Pgp activity.[10]

Several mechanisms involving MDR acquisition have been elucidated.[11] Bebawy *et al.*[12] show sensitive cancer cells acquiring Pgp expression through the transfer of membrane microparticles (MP) derived from resistant cancer cells. MP are small vesicles (0.2–2 μm in diameter) that can be secreted from several cell types.[13] Interestingly, microRNA (miRNA) that may regulate Pgp, and, consequently, MDR, can also be transported in MP and transferred between cells.[14]

Although Pgp protein and mRNA can be transferred to other cells through cancer-derived MP, other molecules that contribute to a resistant phenotype need to be further studied. Here, we evaluate whether the IAP proteins cIAP1, survivin and XIAP can be transported by MP secreted by MDR-positive resistant cancer cells and if they contribute to a multifactorial resistance phenotype in tumor cells. We also investigate the NFκB and Y-Box (Yb-1) proteins, which are oncogenic proteins that can regulate Pgp/ABCB1 and IAP genes expression,[15,16] following co-culture with MP derived from MDR-positive cells.

Material and Methods

Cell lines and cell culture. In this study, we used the human breast adenocarcinoma MCF7, human lung carcinoma A549, and

human CML K562 cell lines, which were purchased from ATCC, and the K562 MDR variant Lucena cell line, as described previously.[17] Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine, and maintained at 37°C under a 5% CO_2 humidified atmosphere. Lucena cells were cultured as previously described.[8]

For co-cultures, sensitive cells were plated on the bottom of 6-well or 24-well plastic culture plates (3×10^5 and 5×10^4 cells, respectively). Cells were cultured for 24 h to ensure sensitive cell adherence, and Lucena resistant cell line (2×10^5 cells/mL) was co-incubated in the same culture medium separated by permeable PET transwell inserts with 3.0-μm pores (Millicell from Millipore - Darmstadt, Germany). Sensitive cells were also co-cultured with isolated MP (described below) from Lucena cells for 24 h.

For conditioned media assays, Lucena cells were cultured without drugs and centrifuged at 1000 *g* for 10 min. Afterwards, conditioned medium was filtered through a 0.22-μm filter (TPP) and added to sensitive cell lines for 24 h.

Membrane microparticles purification. Lucena cells were cultured (2.5×10^8) and used for MP purification by differential centrifugation. First, cells were removed by centrifugation at 1000 *g* for 10 min. To pellet whole cells, the supernatant was centrifuged at 500 *g* for 5 min. Next, the supernatant was ultra-centrifuged (Sorvall RC6+, Thermo) at 30 000 *g* for 20 min at 4°C to pellet the MP. MP were then washed in sterile PBS, and centrifuged as before.

Isolated MP were identified using flow cytometry (FacsScalibur and Accuri, BD) after 15 min of FITC-annexin V staining at room temperature and were also analyzed for protein and RNA content.[18] Fluorescent microspheres of 0.5 and 1.0 μM (Invitrogen, Carlsbad, CA, USA) were used to identify size of MP.

Western blotting and subcellular fractionation. Total cell lysates and western blotting were performed for survivin (R&D Systems, Minneapolis, MN, USA), XIAP (R&D Systems), c-IAP1 (R&D Systems), IκBα (Cell Signaling, Danvers, MA, USA), Akt (Cell Signaling), Phospho-Akt Ser473 (Cell Signaling) and Yb-1 (Abcam, San Francisco, CA, USA) as previously described.[8] The subcellular fractionation analysis of NF-κB (Cell Signaling) and Yb-1 was performed according to the manufacturer's instructions (NE-PER Nuclear and Cytoplasmatic Extraction Reagent Kit; Thermo Scientific, Waltham, MA, USA). To assess Pgp expression (monoclonal anti-Pgp clone C219, 1:10.000), cell lysates were prepared as previously described.[19] Total protein was loaded onto 3–8% gradient NuPAGE Novex Tris-acetate gels (Invitrogen), and proteins were transferred to Hybond-P membranes (GE Healthcare, Buckinghamshire, UK). We normalized the total protein to β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA) and Na^+K^+ATPase (Cell Signaling) and the subcellular fraction to lamina B (Calbiochem - Darmstadt, Germany) and HSC70 (Santa Cruz, Dallas, TX, USA).

To visualize protein expression, we used the ECL detection system according to the manufacturer's instructions (GE Healthcare).

Flow cytometry analysis of P-glycoprotein expression. For Pgp immunodetection, MP derived from Lucena cells, MP derived from parental K562 cells, and recipient cell lines after 24 h of co-culturing (MCF7 and A549) were blocked with 1% BSA for 15 min. Pgp cell surface expression was measured after incubation with an anti-Pgp PE-conjugated monoclonal antibody (clone UIC2; Coulter, Brea, CA, USA) for 30 min through flow cytometry according to the manufacturer's instructions. (FACScalibur, BD or CyAn ADP Analyzer, Dako, Fort Collins, CO, USA).

Determination of P-glycoprotein activity by flow cytometry. To analyze Pgp activity, MCF7 and A549 cells were co-incubated with 200 ng/mL rhodamine-123 (Rho-123) and 200 ng/mL cyclosporine A (CsA) for 45 min at 37°C in a 5% CO_2 humidified atmosphere. Cells were washed in ice-cold PBS and re-incubated with CsA for an additional 45 min under the same conditions. Cells were analyzed by flow cytometry, and the results were expressed as the mean fluorescence intensity ratio (MFI) of cells incubated with Rho-123 and CsA, which was divided by the MFI of cells with Rho-123 alone after subtracting the MFI accounting for auto-fluorescence.

Immunofluorescence. Cells were plated on coverslips, and after 24 h of co-culturing, cells were fixed with 4% paraformaldehyde for 20 min and incubated with 10 mM NH_4Cl for 10 min. The subsequent procedures were performed as previously described.[8] We used anti-Pgp (clone UIC2; Coulter), anti-Yb-1 (Abcam) and anti-NF-κB primary antibodies and Alexa 488-conjugated goat anti-rabbit IgG or Alexa 594-conjugated goat anti-mouse IgG secondary antibodies (Molecular Probes, Eugene, OR, USA). Images were acquired with the NIS-Elements F2.30 software, using an Eclipse E200 Nikon microscope connected to a Digital Sight system.

Apoptosis detection. After 24 h of co-culturing, cells were treated with cisplatin (Accord Farmaceutica LTDA, São Paulo, Brazil), etoposide (Darrow, Rio de Janeiro, Brazil) and paclitaxel (Evolabis, São Paulo, Brazil) for 24 h, in the presence or absence of co-cultured cells. The percentage of apoptotic cells was assessed using the Annexin V/PI assay (Genzyme Diagnostics, Cambridge, MA, USA), according to the manufacturer's instructions.

Real-time quantitative PCR analysis. qRT-PCR was performed to determine *ABCB1*, *Yb-1*, *BIRC5* (survivin), *BIRC2* (c-IAP1) and *BIRC4* (XIAP) mRNA expression levels. Total cellular RNA was isolated using TRIzol (Invitrogen) and mRNA from isolated MP was isolated using the mirVana miRNA Isolation Kit according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). RNA was treated with DNase (Ambion, Carlsbad, CA, USA) to eliminate contaminating DNA, and cDNA was synthesized with the Ready-To-Go T-Primed first-strand kit (GE Healthcare). Gene amplification and analysis were performed as previously described.[8] β-actin was used to normalize C_t values, and all probes were purchase from Applied Biosystems (Carlsbad, CA, USA).

qRT-PCR for miRNA. miRNA from isolated MP and cell lines were extracted using the mirVana miRNA Isolation kit (Ambion, Life Technologies), according to the manufacturer's instructions. Then, miRNA concentrations were quantified using a NanoDrop 1000 (Thermo Scientific), and cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using a primer-specific TaqMan Kit (Applied Biosystems) for hsa-miR-27a, hsa-miR-21 and hsa-miR-451. The miRNA expression levels were normalized to the expression levels of the mature miRNA control RNU6B. These qRT-PCR experiments were carried out using the StepOne Real-Time PCR system.

Statistical analysis. Student's *t*-test or one-way ANOVA were applied for all data analysis. Results were considered statistically significant at $P < 0.05$.

Results

Identification of multidrug resistance chronic myeloid leukemia cell-derived membrane microparticles. Recently, MP from MDR acute lymphoblastic leukemia (ALL) cells have been reported to

mediate the transfer of Pgp to drug sensitive ALL cells.[12] Based on this information, MP derived from CML sensitive cells and a drug-resistant variant were purified and analyzed as described in the Material and Methods. Our results demonstrated that both sensitive and drug-resistant CML cells were able to spontaneously release MP into *in vitro* culture conditions, representing 52.22% and 50%, respectively, of the FITC-annexin V positive population after phosphatidylserine exposure (Fig. 1). We confirmed that MDR cell-derived MP could transport Pgp onto the cell surface. Approximately 22% of the MP-gated population was positive for Pgp. However, MP derived from sensitive cells did not contain Pgp (Suppl. Fig. S1).

Characterization of multidrug resistance chronic myeloid leukemia cell-derived membrane microparticles. In MDR cell-derived MP, we analyzed soluble proteins (cIAP-1, XIAP and survivin) and RNA (mRNA and miRNA) that are involved in the multifactorial resistant phenotype. First, our data revealed that CML MDR-positive cell-derived MP contained anti-apoptotic proteins, such as survivin, cIAP-1 and a small amount of XIAP (Fig. 2a). Their respective mRNA, *BIRC5* and *BIRC2*, but not *BIRC4*, were contained in MP in addition to *ABCB1* mRNA (Fig. 2b).

We also analyzed whether MP could transport miRNA, which are non-coding, small RNA molecules that can induce mRNA degradation or transcriptional repression by binding to complementary 3′UTR sites in the target mRNA and, consequently, regulating gene expression.[20] miR-27a, miR-451 and miR-21 have been shown to be related to Pgp expression and can also contribute to chemotherapy resistance in tumor cells.[21,22] Here, we found that drug-resistant CML cell-derived MP were able to transport miR-451, miR-21 and small amounts of miR-27a (Fig. 2c).

Sensitive cells acquired a resistant phenotype through intercellular transfer of multidrug resistance proteins and RNA. To determine the effect of drug-resistant CML cell-derived MP during resistant phenotype acquisition in non-MDR cell lines, we co-cultured recipient cells (A549 and MCF7) with MDR-positive donor cells (Lucena) for 24 h. Co-culture conditions used Transwell inserts to avoid intracellular contamination and cell–cell contact (see Material and Methods). Initially, our results showed that A549 and MCF7 cells could acquire Pgp protein and mRNA expression (Fig. 3a,b).

Afterwards, we investigated survivin, c-IAP1 and XIAP expression in recipient cells following co-culturing with MDR-positive donor cells. A549 recipient cells clearly showed an increase in all three IAP proteins and mRNA expression after co-culturing. However, MCF7 recipient cells showed only a minor enhancement of XIAP protein expression and no increase of *BIRC2* mRNA expression after co-culturing (Fig. 3c,d). Changes in the mRNA expression profile of A549 and MCF7 cells were confirmed by culturing these cells with isolated MP derived from Pgp-positive cells (Suppl. Fig. S2). Moreover, A549 and MCF7 cells cultured with conditioned media from MDR-positive cells showed no change in Pgp and IAP protein expression levels (Suppl. Fig. S3). These results indicate that MP transfer proteins and mRNA in a cell line-dependent manner.

To evaluate whether acquiring Pgp expression and enhancement of survivin, XIAP and cIAP1 expression could, in fact, induce the MDR phenotype in recipient cells, we tested Pgp efflux pump activity and apoptosis inhibition using chemotherapeutic agents. As shown in Figure 4, A549 and MCF7 recipient cells acquired functional Pgp with little efflux pump activity after co-culturing with an MDR-positive donor cell line (MFI = 1.3 and 1.4, respectively). To analyze apoptosis, A549 and MCF7 cell lines were treated with cisplatin and paclitaxel, respectively, for 24 h after co-culturing with a Pgp-positive donor cell line. The apoptosis index of A549 cells was reduced from 40% to approximately 6% after co-culture with MP (Fig. 4b). MCF7 cells showed a similar reduction, from 30% to approximately 14% (Fig. 4c). These results show that MDR cell-derived MP can induce a drug resistant phenotype in sensitive cell lines.

Oncogenic-related pathways contribute to the multifactorial resistance phenotype following the transfer of multidrug resistance proteins and RNA to sensitive cells. Constitutive activation of the NFκB pathway has been observed in several solid and hematological neoplasms and has been associated with oncogenesis by controlling several key mechanisms, including anti-apoptotic and resistance gene regulation.[23] Our results demonstrated that *BIRC4* mRNA was not contained in MP derived from resistant cells (Fig. 2b). However, we observed *BIRC4* mRNA upregulation in both recipient cells after co-culturing with MDR-positive donor cells and MDR-positive cell-derived MP (Fig. 3c and Suppl. Fig. S2, respectively). Thus, we evaluated NFκB pathway activation through IκBα regulation and NFκB subcellular localization. IκBα levels were reduced in A549 and MCF7 cells after co-culturing with MDR cell-derived MP (Fig. 5a). Moreover, we observed a subcellu-

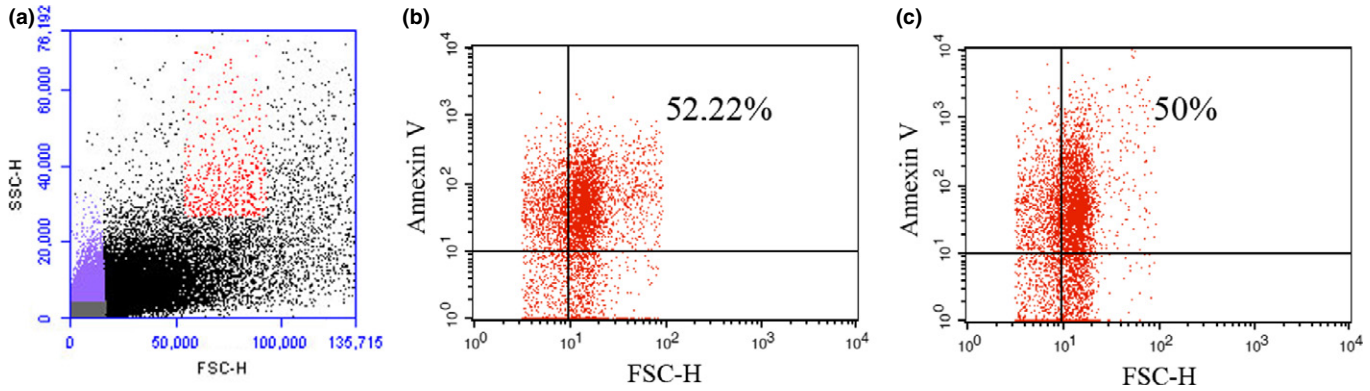

Fig. 1. Cancer cells spontaneously secrete microparticles in *in vitro* culture. Isolated membrane microparticles (MP) were identified by flow cytometry in a dot plot of forward light scattering (FSC) versus side light scattering (SSC). Size of MP was characterized using Fluorescent Microspheres of 0.5 and 1.0 μM. The majority population of cancer cells derived-MP is represented in black and it ranges between 0.5 μM (purple) and 1.0 μM (red) (a). 52.22% of the gated events of K562 cells derived-MP (b) and 50% of Lucena cells derived-MP were FITC-annexin V-positive (c).

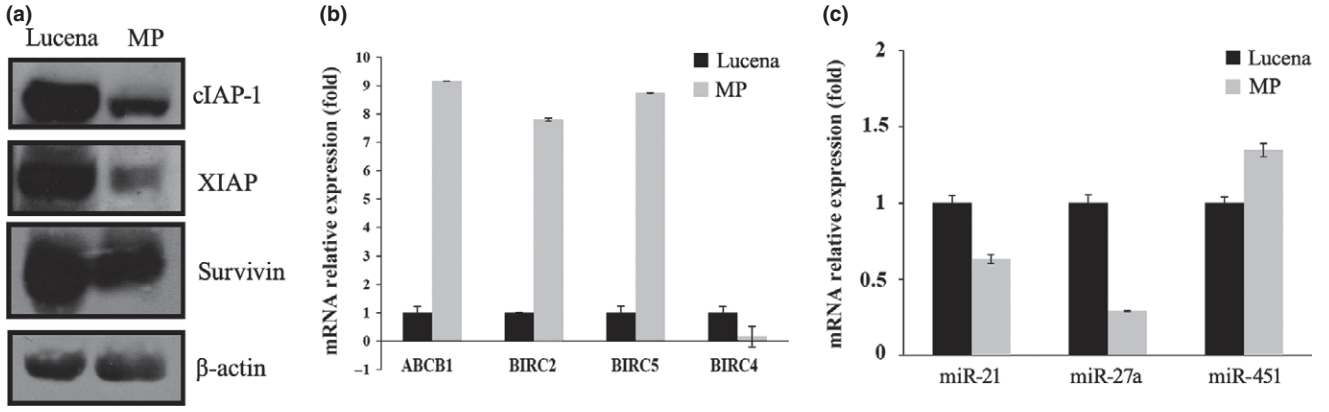

Fig. 2. Microparticles derived from multidrug resistance (MDR)-cells transport resistance proteins and RNA molecules. cIAP-1, survivin and XIAP protein levels of Lucena cells and MDR-positive membrane microparticles (MP) were analyzed by western blot. β-actin was used as load control (a). *ABCB1*, *BIRC2*, *BIRC5* and *BIRC4* mRNA levels were analyzed in Lucena cells and MDR-positive MP by TaqMan quantitative RT-PCR. The mRNA were normalized to β-actin mRNA expression (b). miR-21, miR-451 and miR-27a levels were determined in Lucena cells and MDR-positive MP. These miRNA were normalized to RNUB6 (c). Each bar represents the mean ± SD of triplicate experiments.

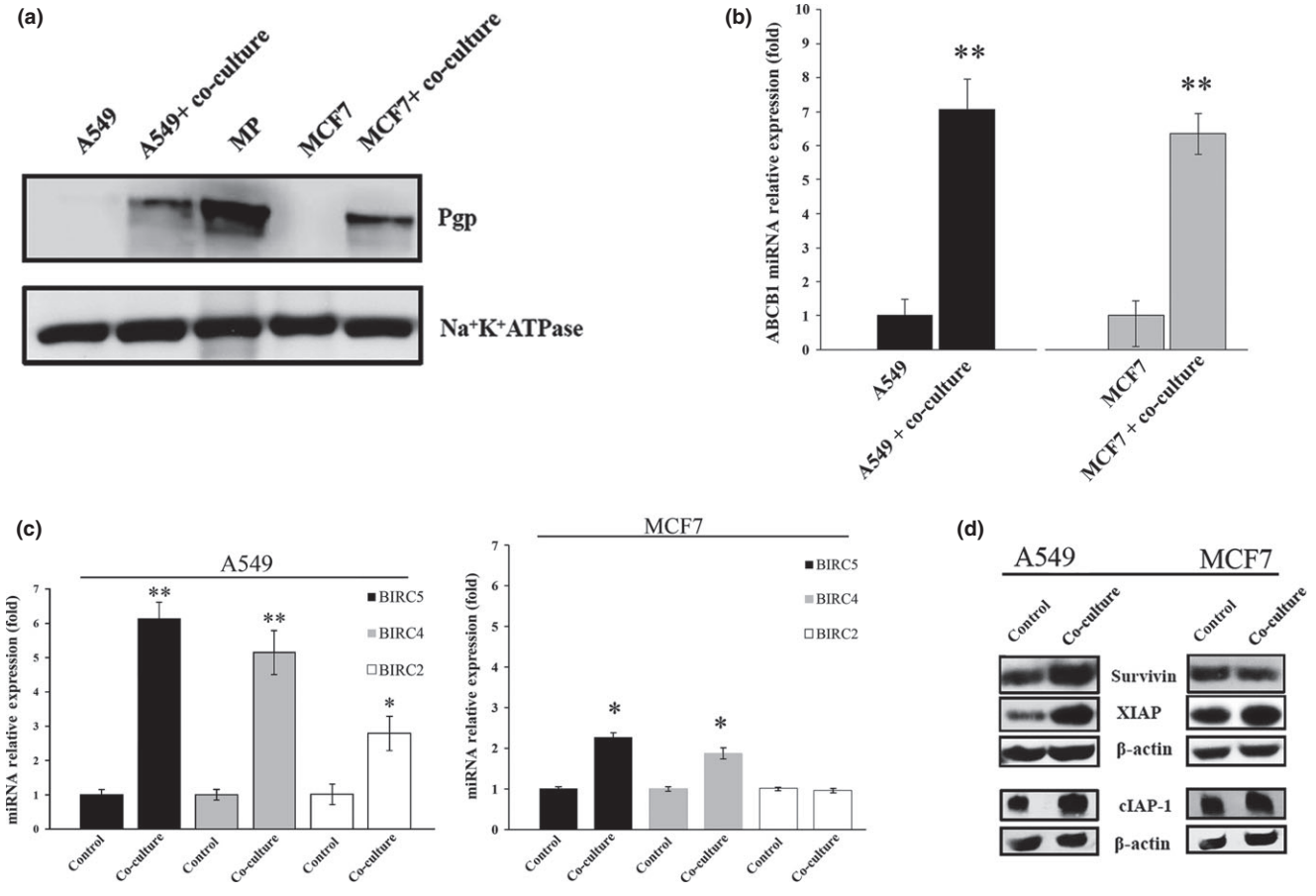

Fig. 3. P-glycoprotein (Pgp) and Inhibtors of Apoptosis Proteins are upregulated in A549 and MCF7 cells after co-culture with multidrug resistance (MDR)-positive cells. Pgp expression was analyzed by western blot – Na^+K^+ATPase was used as load control (a); or quantitative RT-PCR (b). A549 recipient cells showed upregulation of survivin (*BIRC5*), XIAP (*BIRC4*) and cIAP-1 (*BIRC2*) mRNA (c, left graph) and proteins (d, left panel) after co-culturing with MDR-positive cells. MCF7 recipient cells showed an increase of *BIRC5* and *BIRC4* mRNA expression (c, right graph) and a minor enhancement of XIAP protein expression (d, right panel). Quantitative RT-PCR was used for gene analysis and western blot was used for protein analysis. The mRNA were normalized to β-actin mRNA expression and β-actin protein was used as load control. Each bar represents the mean ± SD of triplicate experiments. $*P < 0.05$, $**P < 0.01$.

lar redistribution of NFκB with predominant nuclear localization after co-culturing (Fig. 5b,c). These results strongly suggest the functional NFκB pathway involvement in intrinsic *BIRC4* gene regulation. Likewise, nuclear NFκB may have contributed to intrinsic *ABCB1* and IAP expression in recipient cells (Fig. 3).

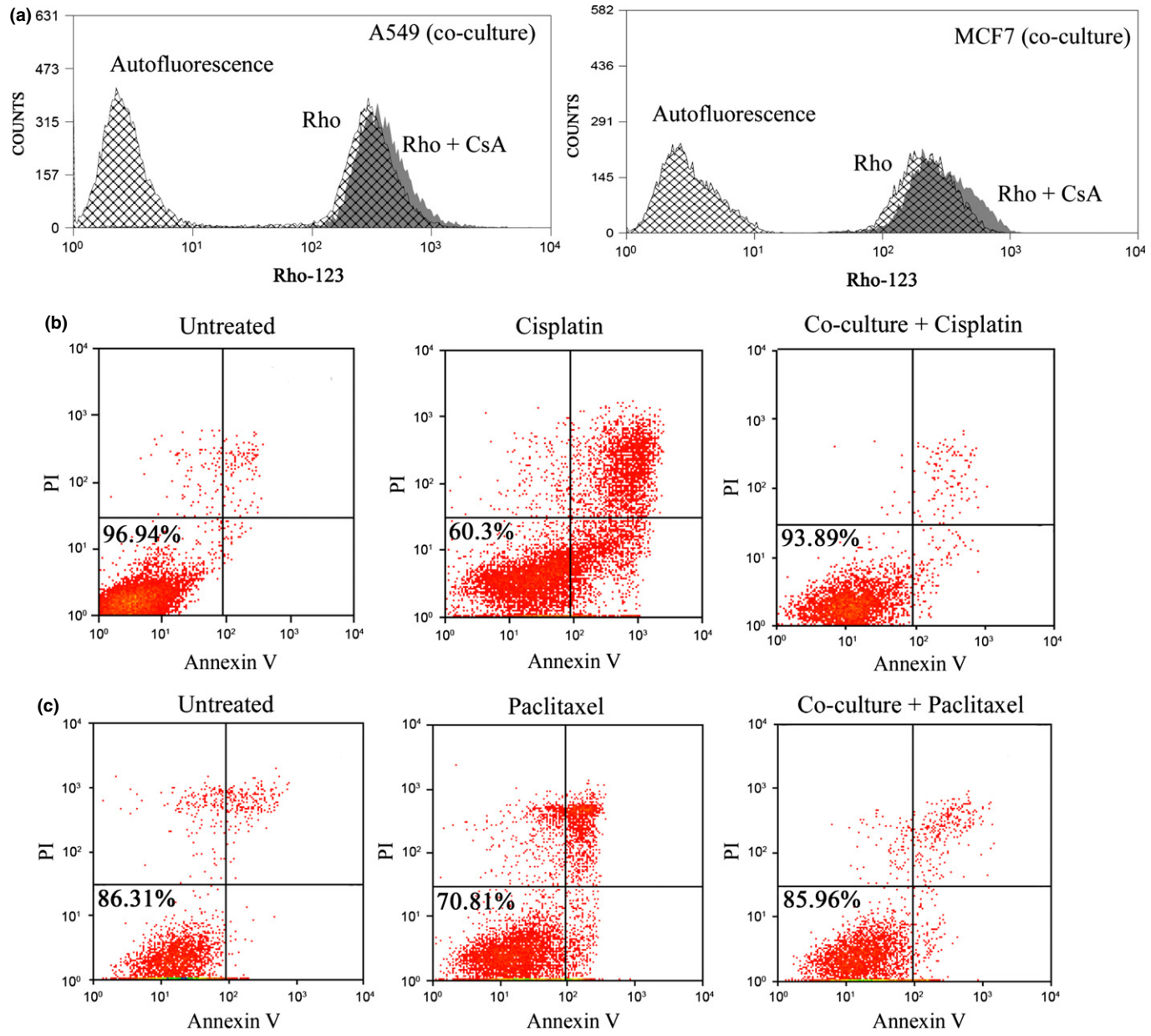

Fig. 4. Multidrug resistance (MDR)-negative cells acquire resistance phenotype after co-culturing with MDR-positive cells. MDR-positive cells transfer a functional Pgp (a) to A549 cells (left panel) and MCF7 cells (right panel). P-glycoprotein (Pgp) activity of A549 (MFI = 1.3) and MCF7 (MFI = 1,4) recipient cells were analyzed by flow cytometry after incubation with rhodamine-123 fluorochrome (Rho-123) with or without cyclosporine A (CsA). The solid gray histogram represents positive activity of Pgp and the hatched black histogram represents cells with Rho-123 (right) or cells alone (left). A549 cells (b) and MCF7 cells (c) were treated with cisplatin and paclitaxel, respectively, for 24 h, and induced cell death was analyzed by flow cytometry using Annexin V and propidium iodide (PI) staining. Cisplatin induced approximately 40% of cell death in A549 cells (middle panel) and only approximately 6% of cell death (right panel) after co-culturing with MDR-positive cells. Untreated cells are represented in the left panel (b). Paclitaxel induced approx. 30% of cell death in MCF7 cells (middle panel) and only approximately 14% of cell death (right panel) in these cells after co-culturing with Pgp-positive cells. Untreated cells are represented in the left panel (c).

We further investigated Yb-1 expression and its subcellular localization in recipient cells. Yb-1 is a multifunctional protein also involved in transcriptional regulation and drug resistance. Yb-1 has been reported to bind to the *ABCB1* promoter region and positively regulate its transcription.[15,24] Although no changes were observed in Yb-1 protein expression (Fig. 5a) and mRNA expression levels (data not shown) after co-culturing with MP, Yb-1 was differentially distributed in recipient cells. We observed a perinuclear subcellular localization of Yb-1 in A549 cells with no change in nuclear and cytoplasmatic localization after co-culturing. However, in MCF7 cells, we detected nuclear foci of Yb-1 after co-culturing (Fig. 5b,d). The nuclear organization in foci of Yb-1 in MCF7 cells suggests Yb-1 participation in *ABCB1* mRNA transcription.

Multidrug resistance cell-derived membrane microparticles transfer oncogenic and resistance miRNA to sensitive cells. Our results showed that MDR cell-derived MP contained miR-27a, miR-451 and miR-21 from donor cells (Fig. 2c). Indeed, we evaluated the transfer of miR-27a, miR-451 and miR-21 from MDR cell-derived MP to recipient cells. Our results showed

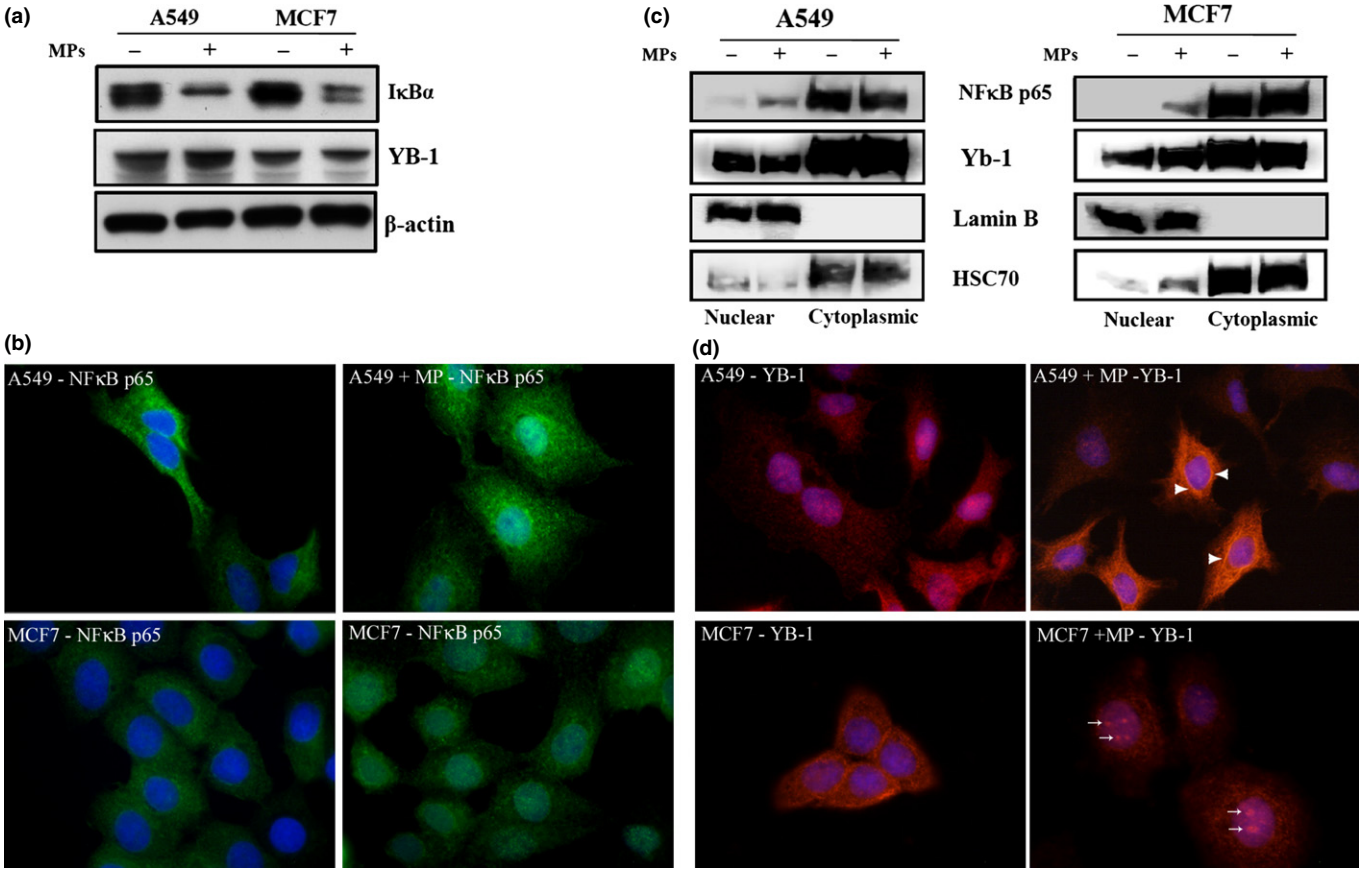

Fig. 5. NFκB and Yb-1 pathway profile are changed in multidrug resistance (MDR)-negative recipient cells after co-culturing with isolated membrane microparticles (MP). NFκB pathway was analyzed in A549 cells and MCF7 recipient cells through IκBα expression levels (a), NFκB subcellular localization by fluorescence microscopy (b), and cellular fractionation (c). Yb-1 pathway was analyzed in both recipient cells through total Yb-1 expression level (a), cellular fractionation (c) and subcellular localization by fluorescence microscopy (d). MDR-negative cells showed degradation of IκBα (a) and NFκB nuclear staining (b), confirmed by NFκB increase in cellular nuclear fractions after co-culturing with P-glycoprotein (Pgp)-positive MP (c). MDR-negative cells, after co-culturing with MDR-positive MP, showed no changes in total Yb-1 levels (a). MCF7 cells showed slight enhancement in nuclear fraction (c, right panel), confirmed by nuclear foci as indicated by arrows (d, lower panel). A549 cells showed a perinuclear staining as indicated by arrowheads (d, upper panel). β-actin was used as load control for total western blot, and lamin B and HSC70 were used as load and purity control of nuclear and cytoplasmatic fractionation, respectively. DAPI was used for nuclear staining (blue), along with polyclonal anti- NFκB staining (green) and polyclonal anti-Yb-1 staining (red). Images captured at 1000× magnification.

that miR-27a and miR-451 were upregulated in A549 and MCF7 recipient cells after co-culturing. In addition, MCF7 cells showed an increase in oncogenic miR-21 expression levels after co-culturing (Fig. 6a,b). These data suggest that miR-27a and miR-451 may contribute to the positive regulation of intrinsic *ABCB1* mRNA expression in recipient cells and that miR-21 may contribute to malignant tumor potential. In addition, we analyzed the Akt activation in both recipient cells, due to the fact that this kinase can be related to NFκB activation via miR-21.[25] After co-culturing, MCF7 showed an increase in phosphorylated Akt protein (Fig. 6c)

Discussion

In this study, we showed that drug-resistant CML cell-derived MP could transfer anti-apoptotic molecules besides Pgp to sensitive breast and lung tumor cells. In addition, we demonstrated that these MP induced changes in the oncogenic phenotype of drug-sensitive recipient cancer cells.

The selectivity of Pgp-positive MP binding was recently discussed by Jaiswal *et al.*, who report that MP secreted from MDR breast cancer cells only transferred Pgp to malignant breast cells. In addition, they showed that MP derived from resistant ALL cells could transfer Pgp to non-leukemic cells.[26] However, Pasquier *et al.*[27] show that MP derived from epithelial and mesenchymal cancer cells interacted with endothelial cells. Here, our results show that MP secreted from drug-resistant CML cells could transfer Pgp/ABCB1 to sensitive breast and lung tumor cells, thus displaying no cell-type selectivity. Moreover, in A549 cancer cells, we observed an increase in survivin, cIAP1 and XIAP protein expression, which suggests that CML cell-derived MP could transfer IAP molecules to lung cancer cells but were unable to transfer these proteins to breast cancer cells. Survivin protein has been previously detected in exosomes (vesicles smaller than MP that are derived from intracellular organelles) secreted from a cervical cancer cell line and from prostate cancer patients.[28,29] Another study using cervical cancer cells showed that cIAP1 and XIAP proteins were enriched in MP after immunoblotting overexposure but not in pure MP fractions.[30] Nevertheless, in this study, we described for the first time the presence of IAP mRNA in CML cell-derived MP.

The transfer of Pgp via MP derived from MDR cancer cells has been reported as a potent mediator of resistance, due to its

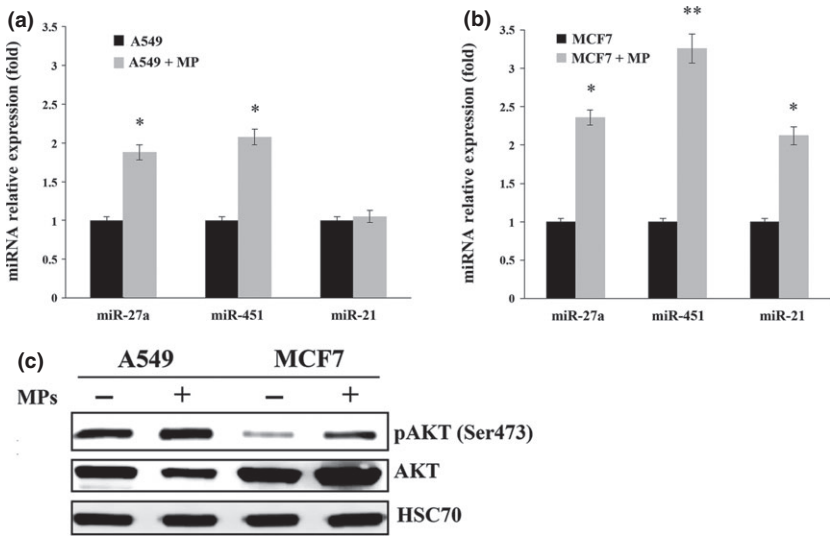

Fig. 6. Changes in microRNA expression profile and activation of Akt pathway in multidrug resistant (MDR)-negative recipient cells after co-culturing with isolated membrane microparticles (MP). miR-27a, miR-451 and miR-21 were upregulated in MDR-negative cells after co-culturing with MDR-positive MP. A549 recipient cells showed an increase of miR-27a and mir-451 expression levels after co-culturing with MDR-positive MP (a). MCF7 recipient cells showed upregulation of miR-27a, miR-451 and miR-21 expression levels after co-culturing with MDR-positive MP (b). MCF7 recipient cells showed activation of phosphorylated Akt (pAkt) (c). Each bar represents the mean ± SD of triplicate experiments. $*P < 0.05$, $**P < 0.01$.

functional drug efflux pump activity.[12] Here, we confirmed that sensitive A549 cells acquired cisplatin and etoposide (data not shown) drug resistance following co-culturing. However, cisplatin is not a chemotherapeutic drug substrate for Pgp,[31] and, thus, evasion of the cisplatin-induced apoptosis displayed by A549 cells must have been acquired through another mechanism. Gong *et al.*[32] propose that drugs can be sequestered by MP, specifically daunorubicin and doxorubicin, and this sequestration might contribute to resistance acquisition. However, considering MP content and transfer ability, we suggest that concomitant expression of IAP and Pgp induces a multifactorial resistant phenotype in A549 cells. These data are supported by our previous study, which showed that XIAP contributed to cisplatin and vincristine drug resistance in CML cells with inhibited Pgp activity.[33] However, MCF7 cells showed paclitaxel-induced apoptotic evasion after co-culturing. Because no IAP protein expression changes were observed in MCF7 cells, other than a slight increase in XIAP levels, we assume that paclitaxel resistance in MCF7 cells results from Pgp expression and activity.

Furthermore, we showed that MDR-positive MP induced changes in the NFκB/p65 expression profile in both sensitive recipient cell lines. NFκB is an oncogenic transcription factor that forms homo/heterodimers, and after stimulation, its nuclear localization can regulate cell differentiation, proliferation and survival. In the canonical pathway, NFκB translocation from the cytoplasm to the nucleus occurs following IκBα phosphorylation and degradation.[34] Here, we demonstrated that IκBα degradation and nuclear NFκB localization occurred in A549 and MCF7 cells after co-culturing. Our data suggest that miR-21 contained in MDR-positive MP may regulate the NFκB pathway in recipient cells via Akt phosphorylation. Iliopoulos *et al.*[35] demonstrated that miR-21 induced cellular transformation and PTEN tumor suppressor gene inhibition, which consequently promoted Akt and NFκB activation. In addition, Pasquier *et al.*[27] showed that cancer cell-derived MP induced Akt phosphorylation in endothelial recipient cells.

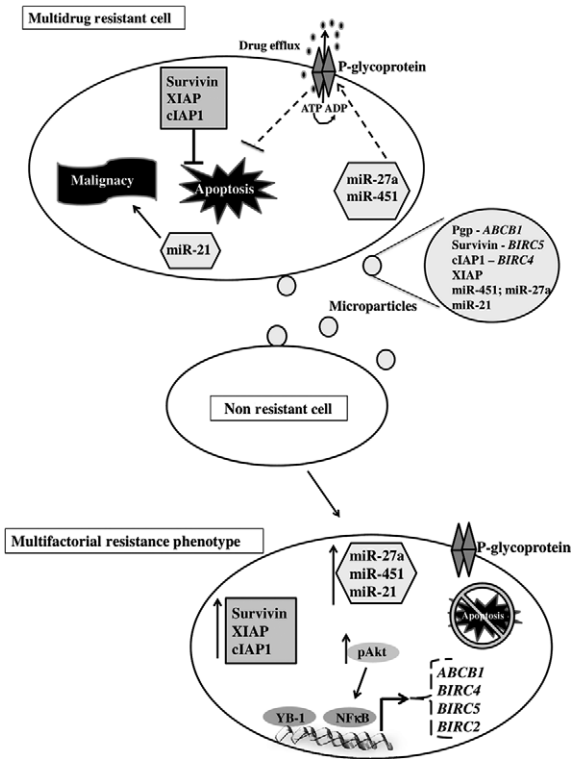

Fig 7. Schematic diagram of multifactorial phenotype acquisition. Multidrug resistant (MDR) cancer cell-derived MP carry and transfer MDR molecules to non-resistant cancer cells, which can then establish multifactorial resistance.

Therefore, we suggest that the NFκB pathway is activated in MCF7 cells via miR-21 transfer by MDR-positive MP. However, no changes in miR-21 expression levels were observed in A549 cells after co-culturing, which indicates that another pathway was activated. However, a variety of miR that have

been associated with NFκB activation can be transferred by MP.[14,36] Indeed, an MP microRNA array would be useful to investigate specific NFκB pathway activation. Nevertheless, we suggest that nuclear NFκB is associated with endogenous IAP and Pgp mRNA expression, mainly *BIRC4*, which was not transported by MP but was upregulated in recipient cells.

We identified changes in the Yb-1 localization profile in recipient cells after co-culturing. Overexpression of the Yb-1 transcription factor has been described in solid and hematological cancers, and its nuclear localization has been associated with a poor prognosis.[37] Here, we observed no changes in Yb-1 expression levels; however, Yb-1 was observed as nuclear foci in MCF7 cells, indicating its transcriptional activation after co-culturing with MDR-positive MP. Chatterjee *et al.*[38] also showed Yb-1 staining in a dotted pattern in multiple myeloma cells, associating Yb-1 with drug resistance and a highly proliferative phenotype. However, A549 cells showed nuclear and cytoplasmatic Yb-1 staining, and after co-culturing, we observed perinuclear Yb-1 staining, which suggests binding to RNA in the endoplasmic reticulum. This hypothesis is supported by Hayakawa *et al.*[39], who showed that Yb-1 could bind to oxidized RNA, most likely to protect it from degradation.

Pgp/ABCB1 regulation has been described to occur through several miR; however, the data regarding miR-27a's regulation of the Pgp and MDR phenotypes are still controversial.[40] Downregulation of miR-27a has been reported in Pgp-positive leukemia cells, and miR-27a transfection has been associated with reduced Pgp expression.[41] Here, we showed small amounts of miR-27a in MDR-positive MP but increased expression levels in recipient cells after co-culturing with MP, which suggest that the acquisition of the MDR phenotype is also associated with miR-27a. Our hypothesis is supported by Zhu *et al.*[21], who demonstrated that miR-27a and miR-451 positively regulate Pgp/ABCB1 expression. Furthermore, inhibition of miR-27a and miR-451 reduced Pgp/ABCB1 expression levels. In support of these results, we also identified an enhancement of miR-451 expression in recipient cells after co-culturing, suggesting that it contributes to the MDR phenotype.

In summary, resistant cancer cell-derived MP carry and transfer MDR and anti-apoptotic molecules to sensitive cancer cells, which can then establish drug resistance. In addition, MP induce the activation of oncogenic pathways and transfer miR, which contribute to the malignant and multifactorial resistant phenotypes of these cells (Fig. 7).

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Microparticles derived from multidrug resistant (MDR) cells transport Pgp proteins.

Fig. S2. Isolated multidrug resistant (MDR)-positive MP transfer *ABCB1*, *BIRC5*, *BIRC4* and *BIRC2* to Pgp-negative recipient cells.

Fig. S3. Conditioned medium (CM) from multidrug resistant (MDR)-positive cells confers no effect on P-glycoprotein and inhibitors of apoptosis proteins (IAP) proteins expression.