Translocation of Intracellular CD24 Towards Cell Membrane Constitutes a Triggering Event for Drug Resistance in Breast Tumor Cell: Correlation With Dynamics of p38MAPK Activation

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Research article

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Abstract

Background

The adaptation capacity of tumor cells to shift dynamically between different states could be responsible for chemoresistance and has been commonly linked to the acquisition of stem cell properties. Here, we have provided new elements concerning the role of the Cancer Stem Cell marker CD24 in the dynamics of tumor cell plasticity and the acquirement of drug resistance in breast cancer.

Methodology

Breast cancer cell lines and cell lineage obtained from Brazilian patients were treated with cytotoxic and non-cytotoxic drugs and the phenotype switching associated with drug resistance was evaluated. Different markers were quantified like the expression of CD24 through microscopy and FACS, the regulation of pro- and anti-apoptotic proteins Bax and Bcl-2, epigenetic changes and MAPK activation using western blotting in wild type and CD24 silencing cells.

Results

The translocation of CD24 from cytosol to cell membrane was a triggering event for the phenotype change of tumor cells exposed to drug stress. Importantly, this phenomenon was observed in all the cells of the different population that was rendered possible thanks to the presence of an intracellular pool of CD24 in each given breast tumor cell. In these CD24⁺ cells, a strong and continuous phosphorylation of the p38 MAPK leading to drug resistance through the tri-methylation of H3K9 and overexpression of the anti-apoptotic protein Bcl-2 was observed. Importantly, the drug-resistant CD24⁺ cells entered into slow-down of cell cycle and restarted to proliferate after several weeks. The reawakened cells presented constitutive p38 activation, higher drug resistance and higher migration capacity. Notably, the use of a p38 activity inhibitor sensitized cells to drug treatment and avoided the appearance of chemoresistance.

Conclusions/significance

In this study, we have associated the cellular localization of CD24 with the changes in the dynamics of p38MAPK activation in breast tumor cells under drug stress. The tandem CD24/p38 plays a crucial role in the acquisition of drug resistance and new cell identity. The use of p38 inhibitor was able to disrupt this partnership and consequently to avoid chemotherapy-induced cell state transition. These results suggest that targeting p38 in breast cancer can overcome adaptive resistance to doxorubicin treatment.

Introduction

Plasticity endows cancer cells with the capacity to shift dynamically between different states which could be responsible for chemoresistance. The adaptation capacity of tumor cells has been commonly associated with stemness when the acquisition of Cancer Stem Cell (CSC) properties by non-CSCs has been reported under different conditions. Indeed, CSCs would be responsible for tumor initiation,

maintenance, metastasis formation, phenotypic plasticity and drug resistance [1–7]. CD24, a cell surface adhesion glycoprotein, was identified as a CSC marker and is present in various types of cancer including breast, pancreatic and lung [8] and has often been associated with more aggressive diseases in ovarian, breast, lung and prostate cancers [9,10]. This explains the considerable interest for this marker in tumor biology and also in treatment outcome. In this line, relevant studies have revealed that the phenotype switching associated with the detection of surface CD24 could be responsible for chemoresistance [11,12]. For instance, CD24 expression level has appeared to be a significant molecular phenotype of cisplatin-resistant residual cells in laryngeal carcinoma lines which corresponds to a differential expression of critical apoptotic and drug resistance [13]. Goldman *et al.* have observed an enrichment of CD24⁺ cells following treatment with docetaxel in different breast tumor cell lineages, which corresponded to the generation of new CSCs from non-CSCs [12]. Importantly, the mechanism leading to an increased surface CD24 expression in tumor cells under drug stress remains unknown.

Recent findings support the hypothesis that epigenetic mechanisms are key players in the phenotypically transition of tumor cells [14,15]. Epigenetic can result in drastic changes not only in cell plasticity but also in drug resistance [16,17]. Besides, MAPK signaling pathways, which are deregulated in tumor [18,19], have been constantly associated with chemoresistance in different type of cancer [20–22]. For instance, it has been reported that p38 and JNK MAPK pathways play a role in the control of the balance of autophagy and apoptosis in response to genotoxic stress [20]. Others have explored the role of ERK and p38 MAPK in breast cancer chemotherapy [21]. But, little is known concerning the involvement of MAPK in tumor plasticity. Indeed, the molecular mechanisms that control cellular plasticity upon drug treatment remain to be fully established.

Here, using different breast cancer cell lines, we have evaluated the impact of drug stress on the immediate phenotype change. We have shown that the rapid translocation of the CSC marker CD24 from cytosol to cell membrane was the triggering event for the acquisition of chemoresistance. In drug-resistant MDA-MB-231 cells, we have identified a tandem constituted by CD24 and MAPK p38, where the continuous p38 activation controlled the tri-methylation of H3K9 and the overexpression of the survival marker Bcl-2. This phenotype enabled the cells to enter in slow-down of cell cycle, after which several weeks later, the dormant cells retrieved their capacity to proliferate, and were characterized by an increased resistance to drug and migratory capacity. Importantly, the use of p38 inhibitor was able to block the acquisition of drug resistance by impeding the upregulation of the anti-apoptotic protein Bcl-2. The treatment with the TLR7 agonist Imiquimod, which reduced cell proliferation without affecting phenotype switching neither Bcl-2 expression allowed confirming a direct correlation between translocation of CD24 and increased expression of Bcl-2. Finally, we propose that the association of the p38 inhibitor, SB203580, with doxorubicin, a drug leader in clinic, could open up a new strategy in the fight with cancer.

Materials And Methods

Reagents

Thiazolyl Blue Tetrazolium Blue (MTT), Doxorubicin, Staurosporine and RIPA buffer were purchased from Sigma-Aldrich. p38 inhibitor SB203580 and Imiquimod were obtained through InvivoGen. Protease cocktail inhibitor and phosphatase cocktail inhibitor (Roche) were acquired from Sigma Aldrich. Monoclonal antibodies against phospho-p44/42 (ERK1/2), phospho-p38 MAPK, p38 MAPK, cyclin D1, b-tubulin, b-actin and peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Cell Signaling Technology and anti-Bcl-2 (Novex) and anti-Bax (Invitrogen) from Thermo Fisher Scientific. Polyclonal antibodies anti-Histone H3 (tri-methyl K9) and anti-Histone H3 (Acetyl K9) were purchased from Abcam; Apoptosis, DNA Damage and Cell Proliferation Kit from BD Biosciences and Lipofectamin 300 transfection reagent (Invitrogen) from Thermo Fisher Scientific. SiRNAs for CD24 silencing (Cat# Hs01 00232148) and for control (Cat# SIC001) were synthesized by Sigma-Aldrich. PVDF membranes (0.45mM) and Luminat (Millipore) were acquired from Merck and nitrocellulose membrane (0.22mM) from Bio-Rad.

Cell Culture Conditions

We obtained the breast tumor cell lines MDA-MB-231 from ATCC (USA). MACL-1 and MGSO-3 were obtained from Brazilian patients with breast cancer [51]. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS). For starvation conditions, cells were incubated with serum-free DMEM for 2h before the addition of the respective stimuli.

Obtaining of the resistant cell clones from parental MDA-MB-231

The procedures to obtain MDA-MB-231 cells resistant to doxorubicin (named CD24⁺/DxR cells) are schematized in figure 4a. Briefly, MDA-MB-231 cells were incubated with doxorubicin at 0.6mM for 48h. The remaining cells, named CD24⁺/DxR, were trypsinized and used in the respective experiments. These cells were characterized as non-proliferative. DxR/30 achievement is schematized in figure 5a. After obtaining CD24⁺/DxR cells, these cells were cultured with DMEM 10% of fetal bovine serum for 15 days, then cells were cultured under stress condition (DMEM 1% of fetal bovine serum for 48h) following by incubation in normal culture conditions until they recover their proliferative activity. From then, cells were named DxR/30. In order to obtain MDA-MB-231 cells resistant to Imiquimod, MDA-MB-231 cells were incubated with Imiquimod at 1mg/ml for 96h. The remaining cells were trypsinized and used in the respective experiments.

MTT (viability assay)

For doxorubicin assay, MDA-MB-231 cells (10⁴/well) were plated in 96-well plates in DMEM with 10% serum. Cells were incubated for 24h with different stimuli according to the figures. For Imiquimod assay, MDA-MB-231 cells (10⁴/well) were plated in 24-well plates in DMEM with 10% serum. Cells were incubated for 96h with different stimuli according to the figures. After the respective treatment time, the medium was removed and cells were incubated with 50ml/well of DMEM 10% FBS plus of 40 ml/well of

MTT (5mg/ml) for doxorubicin assay or ml/well of DMEM 10% FBS plus of 40 ml/well of MTT (5mg/ml) for Imiquimod assay for 2h at 37°C. After removing the medium, 50ml (for doxorubicin) or 200 ml (for Imiquimod) of DMSO were added to dissolve the crystals of formazan and the absorbance was measured in a microplate reader at a wavelength of 595nm. A value of 100% was assigned to untreated control cultures. Results were derived from at least three independent sets of triplicate experiments.

Histone extraction

After harvesting and washing with ice-cold PBS (twice), cells were resuspended in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN₃) at a cell density of 10⁷ cells per ml. Cells were lysed on ice for 10 minutes with gentle stirring. Then, cells were centrifuged at 2000rpm for 10 minutes at 4°C and the supernatant was discarded. In the next step, cells were washed in half the volume of TEB and centrifuge at before. The pellet was resuspended in 0.2N HCl at a cell density of 4x10⁷ cells per ml over night at 4°C. Then, samples were centrifuged at 2000rpm for 10 minutes at 4° and the aliquots were stored -20°C.

RNA silencing

MDA-MB-231 cells were seeded in 24-well plates at a density of 2x10⁴ cells/well. After 24h of incubation, the cells were transiently transfected with short interfering RNA (siRNA) specific for CD24 or control siRNA at a final concentration of 100nM by using Lipofectamin 3000 Transfection Reagent according to manufacturer's instructions. Briefly, siRNA and Lipofectamin were diluted separately in serum-free OPTI MEM. Then, the diluted Lipofectamin and siRNA were mixed (1:1 v/v). After 5min, the mix was added drop-wise onto the cells under their normal growth conditions and after 48h silenced cells were used in the different assays. CD24 silencing was confirmed by western blotting.

Western Blotting

For protein expression, cells were harvested, counted and lysed in RIPA buffer supplemented with phosphatase and protease inhibitor cocktail according to the manufacturer's instructions. For the evaluation of the phosphorylated form of MAPKs, cells were seeded in 6-well plates (10⁶ cells/well) and were starved for 2h in free-serum medium (SFM). Then, cells were treated for 30~60 min with medium containing 10% FBS as stimuli. At the end of treatment cells were lysed in RIPA buffer supplemented with phosphatase and protease inhibitor cocktail according to the manufacturer's instructions. Protein lysates were separated by polyacrylamide gel electrophoresis on 10% or on 15% gels when concerned histone extract, and electrotransferred to PVDF-0,45mM membranes (Milipore) or nitrocellulose-0,22mM (BioRad) when concerned histone extracts. Membranes were blocked overnight with 5% dry milk and were incubated with primary antibodies in 5% BSA also overnight. After incubation with the peroxidase-conjugated secondary antibody for 1h, protein expression was detected using Luminat HRP reagent (Milipore) and analyzed using LAS-4000 imaging system (Fuji) or C-DiGit Blot Scanner (Li-Cor).

Magnetic sorting

The different cell subpopulations (CD24⁺ and CD24⁻) were sorted from parental MDA-MB-231 cells using *CD24 MicroBead Kit (MACS Miltenyi Biotec)* following manufacturer's instructions. Briefly, the CD24⁺ cells were indirectly magnetically labeled with CD24-Biotin antibodies and Anti-Biotin MicroBeads. Then the cell suspension was loaded onto a MACS Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD24⁺ cells were retained within the column. The unlabeled cells (CD24⁻ cells) ran through; this cell fraction was thus depleted of CD24⁺ cells. After removing the column from the magnetic field, the magnetically retained CD24⁺ cells can be eluted as the positively selected cell fraction. The purity of the sorted populations was verified by Flow Cytometry.

Flow cytometry analysis

To evaluate the proliferative activity of cells, an assay using the Apoptosis, DNA Damage, and Cell Proliferation Kit from BD Biosciences was realized in accord with the manufacturer's instructions. Cells were cultured as indicated in the figure legends and washed/blocked in staining buffer (PBS 4% fetal bovine serum - v/v). Cells were fixed and permeabilized using *CitoFix/CitoPerm* reagent for 20 min on ice, nucleus was permeabilized using *CitoFix/CitoPerm Plus* for 10 min on ice and finally treated with DNase (30mg/10⁶cells) for 1h at 37°C in humidified chamber. Then, cells were simultaneously stained with anti-BrdU/PerCP-Cy5.5 and anti-gH2AX/Alexa647 (BD Biosciences) fluorescent antibodies for 20 min at room temperature. Between every step cells were washed with 1 x PBS/PermWash (BD Biosciences). Concerning CD24 and p38 staining, the following step by step was realized. For extracellular staining, MDA-MB-231 cells were washed/blocked in staining buffer (PBS 4% fetal bovine serum - v/v) and were labeled using anti-CD24/Pe-Cy7 (eBioscience) or anti-CD24 (BD Pharmigen) for 20 min on ice, and when necessary, followed by FITC-conjugated equivalent secondary antibody (BD Pharmigen) for more 20 min on ice. For intracellular staining, after extracellular labeling, cells were fixed and permeabilized using *CitoFix/CitoPerm* reagent for 20 min on ice. Then, cells were incubated with anti-CD24/Pe-Cy7 (eBioscience) and/or with anti-pp38 (Cell Signaling) for 20 min at room temperature, and when necessary, followed by FITC-conjugated secondary antibody (BD Pharmigen) for 20 min at room temperature. Between every step cells were washed with 1 x PBS/PermWash (BD Biosciences). After labeling protocols, cells were fixed in PFA 4% overnight (4°C lightless). Cells were re-suspended in isoton buffer and analyzed by flow cytometry. Single-stain controls were used to set gating parameters and any compensations. All flow cytometry results were analyzed by FlowJo software following a rigorous doublet discrimination based on FSC:A versus width as well as FSC:A versus height. Data were collected by the cell analyzing LSRFortessa (BD Biosciences – Immunocytometry Systems) using “[BD FACSDiva™ Software](#)” (BD Biosciences) and analyzed with “FlowJo (Tree Star) Software”.

Wound Healing Assay

MDA-MB-231 and DxR/30 cells were seeded (8x10⁵) in 6 wells plate and let in growing conditions (DMEM 10%FBS, at 37°, 5%CO₂) for 24 hours or until cells get confluent. Then, using a 100ml tip, a scratch was performed in the cell monolayer and cells were cultured in DMEM supplemented with 2% of FBS in order

to avoid cell proliferation. To obtain the same field during the image acquisition, pen markings were performed at the bottom of the culture plates as reference points close to the scratch. Cell migration was registered by light microscopy (Evos Skedda) at 24, 48 and 72 hours post scratch. Using ImageJ software the wound healing was measured and a closing-time percentage was calculated based on the initial scratch size of each cell type.

Statistical analysis

The data were presented as mean of triplicates $\pm$ SD or as means of triplicates. Statistical significance was determined using Student's t-test, or Two-way ANOVA followed by Bonferroni post-test. The criterion for statistical significance was $p < 0.05$.

Results

1 – The translocation of CD24 from cytosol to membrane is an early event in breast tumor cells under drug stress

Phenotype switching, also commonly referred to as cell plasticity, is an important process observed during treatment of cancer which was repeatedly associated with stemness. Here, using breast cancer cell line, we explored the dynamics of the CSC marker CD24 after doxorubicin treatment. At first, we sought to define the localization of CD24 in MDA-MB-231 cells by extra and intracellular staining. As shown by our results obtained by flow cytometry (Fig 1a,b) only about 5% of cells expressed CD24 in cell membrane corroborating with other studies [23] which explains why MDA-MB-231 is considered as CD24^{low/-}. By contrast, a significant intracellular pool of CD24 was encountered in all cells. Fluorescence microscopy confirmed the presence of both extracellular (yellow arrows) and intracellular CD24 (white arrows) (Fig 1c). After the treatment with doxorubicin at 0,6mM – concentration representing the EC50 after 24h treatment calculated in MDA-MB-231 cell line – a cell phenotype switching occurred which corresponded to an enrichment of the CD24⁺ subpopulation (Fig 1d). Notably, MFI analysis showed an increase of surface CD24 density during drug treatment (Fig 1e). This phenomenon occurred rapidly since ~42% of cells converted into CD24⁺ after 2h to finally reach ~96% after 48h of treatment, as visualized by flow cytometry (Fig 1d). Importantly, the majority of cells remained positive even after a pause in the treatment (incubation in drug-free medium for 48h after treatment) as visualized by the last pseudocolor plot in the figure 1d. The fact that this event was detected in the first hours of treatment excludes the possibility of a Darwinian selection of CD24⁺ cells. These results led us to hypothesize that the intracellular pool of CD24 immediately available might play a role in this process by translocating from the cytosol toward cell surface under doxorubicin treatment. To support these data, MDA-MB-231 cells were sorted into CD24⁺ and CD24⁻ subpopulations (Fig 1h) by using magnetic beads and the CD24 localization was evaluated in CD24⁻ cells after doxorubicin treatment. As shown in the figure 1i, the translocation of CD24 occurred even in CD24⁻ population obtained after cell sorting since CD24⁻ cells were able to rapidly convert in CD24⁺ cells. Such data reinforce the idea that CD24⁺ cells enrichment

during drug treatment does not correspond to a pre-selection of clones but to a drug-induced phenotype switching. In order to confirm this theory, we took the opportunity of using brefeldin A, an inhibitor of protein transport from endoplasmic reticulum to Golgi apparatus, to disturb the CD24 traffic after drug treatment. After flow cytometry analysis, we observed that when MDA-MB-231 cells were treated with brefeldin A before doxorubicin, the translocation of CD24 was reduced (Fig 1f). These results were consistent with the fluorescence microscopy images obtained from doxorubicin-treated cells and stained with anti-CD24 without permeabilization to solely detect surface CD24 (Fig 1g). To discard the hypothesis that the increased membrane CD24 expression could be partially due to an increased protein synthesis, we used actinomycin D, a DNA-transcription inhibitor. The incapability of actinomycin D to reduce the CD24⁺ phenotype enrichment in presence of doxorubicin indicated that the intracellular pool of CD24 was the main source of CD24 traffic in the presence of doxorubicin (data not shown).

Concerning MACL-1 and MGSO-3 breast cell lines, obtained from Brazilian patients, the percentage of CD24⁺ cells was about 5% and 46% respectively, corroborating with their classification in other study [24]. The presence of intracellular CD24 was also detected in the whole population of both cell lines, like observed in MDA-MB-231 population (Fig 2a,b). As observed in MDA-MB-231 cells under treatment, phenotype switching occurred in both cell lines which corresponded to an enrichment of the CD24⁺ subpopulation (Fig 2c,d) since ~27% and ~45% of MACL-1 and MGSO3 cells, respectively, converted into CD24⁺ after 4h of treatment with doxorubicin. After 24h of treatment, the conversion rate in CD24⁺ cells reached ~90% for MDA-MB-231 and MACL-1 and ~70% for MGSO-3 cells.

Therefore, we propose a new dynamic model of cell transition phenotype under drug stress that could allow each cell of breast cancer to convert into CD24⁺ cells which involves the translocation of intracellular CD24 accompanied with its increased expression at cell surface.

2 – Translocation of CD24 correlates with the upregulation of Bcl-2 expression leading to chemoresistance in MDA-MB-231 cells treated with doxorubicin

Here, we investigated the molecular identity of the survival cells after 48h of doxorubicin treatment named CD24⁺/DxR cells. The figure 3a represents the methodology used to obtain CD24⁺/DxR cells and schematize the steps to perform the following MTT and western blot assays. At first, we tested the sensibility of the CD24⁺/DxR cells to respond to a second doxorubicin treatment using MTT assay. As shown in the figure 3b, only a slight reduction of CD24⁺/DxR cell number was noted after a second treatment with doxorubicin while the viability of naïve cells, that received the treatment for the first time, declined below 40%. These data indicated that CD24⁺/DxR cells became tolerant. So, we sought to identify whether this phenotype change corresponded to a putative cell reprogramming in response to drug stress. By using western blot assay, we compared the protein profile of naïve and CD24⁺/DxR cells. When we focused on protein expression involved in cell proliferation or death, it was detected a significant increase of Bcl-2 expression which was inversely correlated with Bax expression in CD24⁺/DxR (Fig 3c). In addition, a remarkable decrease of cyclin D1, a regulator of cell cycle progression, was noted,

suggesting that acquisition of drug tolerance controlled by Bcl-2 expression may also require cells to exit the cell cycle (Fig 3c). In previous studies, we have focused on the role of p38 MAPK and ERK1/2 in the proliferation of MDA-MB-231 cells [25,26], so we analyzed their activation profile in the resistant CD24⁺/DxR cell. Interestingly, this phenotype switching was accompanied by a strong and continuous activation of p38 MAPK at the detriment of another MAPK, ERK1/2 (Fig 3d). Then, we verified the relationship between CD24 and Bcl-2 by silencing CD24 using interference-RNA. In CD24-silenced cells (SiCD24), a decreased Bcl-2 and p38 expression was observed (Fig 3f). This may explain the reduced capacity of SiCD24 cells to resist to drug treatment in all the doxorubicin concentrations tested when compared with control-silenced (SiC) and parental MDA-MB-231 cells (Fig 3e). By contrast, CD24⁻ subpopulation, obtained from magnetic sorting, presented a similar drug sensibility when compared with CD24⁺ subpopulation and parental MDA-MB-231 cells, probably due to the phenotype switching after drug treatment thanks to CD24 translocation as shown in the figure 1j (Fig 3e).

Next, we sought to treat MDA-MB-231 cells with a drug capable to reduce cell proliferation without inducing immediate death, like doxorubicin does. We tested the efficacy of the TLR7 agonist Imiquimod, previously used in skin cancer treatment and in the treatment of cutaneous metastatic breast cancer [27–29], on MDA-MB-231 cell proliferation. At the concentration of 1mM, a significant decreased in cell proliferation was observed while a total blocking of cell replication was noted at 10mM during the period of experiment (Fig 3g). No significant cell death was observed in the first 48 hours in the presence of both concentrations of Imiquimod. In such context, we tested the capacity of cells to respond to a second treatment 96h after the first dose of Imiquimod. As shown in the figure 3h, where the results are expressed in % of cell viability, we observed a similar pattern when an unique dose or two subsequent treatments with the TLR7 agonist were used indicating that no phenotype change occurred after the first treatment and consequently avoid chemoresistance. When we used CD24 as a phenotypic marker, we confirmed that no switching phenotype of MDA-MB-231 population leading to an enrichment of CD24⁺ cells occurred (Fig 3i). In accord with this, no upregulation of Bcl-2 expression was detected in cells treated with Imiquimod (Fig 3j).

Taken together these data indicated that translocation of CD24 is a triggering event leading to phenotype change and upregulation of Bcl-2 expression.

3 – CD24 and p38 work in tandem in the chemoresistance acquisition phenotype in breast cancer cells.

According to the figure 3d, p38 phosphorylation was stronger, constitutive and independent on serum in CD24⁺/DxR cells contrasting with the serum-dependent activation of p38 in MDA-MB-231 cells under normal culture conditions. This suggests that p38 activation in different configurations can cause different outputs and may participate in the phenotype switching.

The next question was whether there was a privileged relationship between CD24 and p38. At first, we explored this point in MDA-MB-231 cells under growing culture conditions. After magnetic sorting, we evaluated by western blot the status of MAPK activation after cell stimulation with serum according to

the kinetic presented in the figure 4a. As clearly shown, CD24⁺ cells phosphorylated p38 in a more pronounced way than CD24⁻ subpopulation. In contrast, a higher phosphorylation of ERK1/2 was observed in the CD24⁻ and parental MDA-MB-231 cells. The results obtained by flow cytometry confirmed the correlation between surface CD24 expression and preferential p38 phosphorylation, as observed by the fact that ~70% of the CD24⁺ cells phosphorylated p38, while in only ~15% of the CD24⁻ cells the activation of this MAPK was observed (Fig 4b).

Another evidence demonstrating that CD24 and p38 work together is presenting in the figure 4c. According to the western blotting, siRNA-mediated knockdown of CD24 decreased the phosphorylation of p38 when cells were submitted to doxorubicin treatment indicating that the absence of CD24 jeopardized the cell capacity to induce activation of p38 MAPK.

The sustained p38 activation in CD24⁺/DxR cells makes it a prime target. In this context, we used SB203580, a p38 activity inhibitor [30] to evaluate its impact on drug resistance. According to the results obtained by cell counting, the combination of SB203580 and doxorubicin was more efficient in reducing cell number than doxorubicin alone (Fig 4d). Concerning the results observed by MTT assay, the inhibition of p38 was benefit from two aspects: firstly, SB203580 sensitized MDA-MB-231 cells to the therefore doxorubicin treatment (blue line vs black line) and at second, SB203580 disrupted the resistant-phenotype acquired by the cells that received two consecutives doxorubicin treatments (green line vs red line). The effect of the drug association may be considered as synergistic since the total *effect* of the combination of SB203580 and doxorubicin was greater than the sum of the individual *effects* of each *drug*. *Importantly*, SB203580 alone was unable to impact on cell viability in the same experimental conditions (Fig 4e).

To visualize these results, we performed the capture of light microscopy images of cells treated with drug pair. These experiments were performed under sub-confluence or confluence conditions to exclude the influence of fluctuating environment. A direct impact of doxorubicin on MDA-MB-231 cells was observed after 24h treatment marked by a decreased cell number and changes in morphology. The association of SB203580 and doxorubicin exacerbated the cell phenotype changes under confluent and sub-confluent conditions. The results confirmed that the efficiency of the drug pair constituted by doxorubicin and SB203580 was superior in killing cells in both plating conditions (Fig 4g).

Consistent with the above results, western blots showed that SB203580 prevented the augment of Bcl-2 expression induced by doxorubicin (Fig 4f). Further, in MDA-MB-231, which has high levels of a mutant p53, it has been described that p53 mutants can contribute to the suppression of apoptosis [31]. In line with this, SB203580 was also able to reduce the expression of p53 in doxorubicin treated cells (Fig 4f).

Epigenetic events drive cell reprogramming and tumor cell plasticity [14,15]. In this context, we have, evaluated whether the state of histones could be altered in CD24⁺/DxR cells. We focused on the lysine 9 at the histone H3 (H3K9), which can turn genes on or silence them by getting acetylated or methylated [32]. Our western blot analyzes have shown that doxorubicin treatment increased the tri-methylation of

H3K9 (H3K9me3) which combined with its deacetylation. Importantly, SB203580 was able to prevent the increase of H3K9me3 induced by doxorubicin treatment indicating that p38 can regulate the methylation state of H3K9 observed under drug pressure (Fig 4h).

Taken together, these results suggest that targeting p38 during the chemotherapy-induced phenotypic cell state transition can overcome adaptive resistance to doxorubicin treatment.

4 – CD24/DxR cells become proliferative after a long-lasting period in dormancy.

The capacity of slow cycling cells to reentry into cell cycle has been a topical debate for quite a time. As reported above, CD24⁺/DxR cells have adopted a slow-down cell cycle after doxorubicin treatment evidenced by a reduction of cyclin D1 (Fig 3c) and were characterized by an enlarged morphology that are hallmarks of dormant cells (Fig 5b).

So, we sought to monitor CD24⁺/DxR cells to evaluate the reversibility of their dormant state according to the scenario presented in the figure 5a. CD24⁺/DxR cells were cultured in drug-free medium for a long period and then were submitted to a serum deprivation for two days followed by the culture in medium contained 10% of serum. In such conditions, we observed the emergence of revertant cells (named DxR/30) which reacquired the ability to proliferate as confirmed by their capacity to incorporate BrdU (Fig 5c).

The percentage of CD24⁺ cells in the revertant-population recovered to levels observed in naïve MBA-MB-231 cell population (Fig 5d). Importantly, even in the absence of drug, the phosphorylation of p38 in DxR/30 remained strong and constitutive indicating that DxR/30 cells have conserved some features of their precedent states while having eliminated others (Fig 5e).

When we evaluated the drug resistance of DxR/30 cells, more than one month after the first treatment the cells remained tolerant to doxorubicin. Indeed, as shown in the light microscopy images, the morphology of these cells appeared little affected after treatment when compared to naïve MDA-MB-231 treated cells. More surprisingly, DxR/30 cells retained their capacity to proliferate even in the presence of drug (5 days) without entering in slow cycling stage like CD24⁺/DxR, confirming that DxR/30 cells have acquired a new identity. After 12 days of treatment, they have re-colonized the plastic dishes (Fig 5f). One of the hypothesis is that DxR/30 cells might have a competitive advantage over naïve cells under drug stress due to their constitutive phosphorylation of p38. Finally, we evaluated the migratory capacity of these cells by using the *in vitro* wound healing assay based on the creation of an artificial gap, so called "scratch", on a confluent cell monolayer. Images were captured every 24 hours during cell migration, the scratch was measured and a comparison of time required to close the scratch between naïve cells and DxR/30 was performed. The incubation time was determined at 48h when the faster moving cells DxR/30 were just about to close the scratch. The confirmation of the migratory capacity of both cells was made by using the software Image J (Fig 5g).

According to our data, slow-cycling cells under stress may reentry into cell cycle leading to cells that possess new properties, including higher drug resistance and higher migratory capacity, reaffirming that they have acquired a new identity.

Discussion

Tumor cells have been shown to hijack signaling pathways involved in reprogramming to become phenotypically plastic and evolve towards drug-refractory cell identities. Our results provide new insight about these mechanisms leading to the emergence of resistant subpopulation during chemotherapy treatment. Robust evidence showed that the CSC subpopulation is enriched after chemotherapy, suggesting that this subset is responsible for the majority of treatment failure [33,34]. Here, by monitoring the CSC marker CD24 during doxorubicin treatment, we have shown the uniform conversion of CD24⁻ breast cancer cell population into CD24⁺ cells characterized as drug resistant cells. In the same line, Goldman *et al* have reported that the treatment of breast or ovarian cancer cells with high concentration of taxanes results in the generation of 'persistent' cells, which are defined by a transition towards a CD44^{Hi}CD24^{Hi} expression status [12]. Others studies have demonstrated an enrichment for CSC-like phenotype after chemotherapy in glioblastoma [35] indicating how the phenomenon is ubiquitous.

Importantly, our data revealed that each given breast tumor cell may convert into CD24⁺ phenotype thanks to the translocation of CD24 from cytosol to cell membrane made possible by the presence of an intracellular pool of CD24, never described until now. Such findings suggest that CSC may not be considered as a clonal identity but rather a state as also claimed by Dirske *et al* [35]. The conversion observed between non-CSC and CSC after chemotherapy demonstrates that cell plasticity emerges as an important contributor to therapy escape. This was verified when we used the TLR7 agonist Imiquimod, capable to reduce MDA-MB-231 cell proliferation without inducing translocation of CD24⁺, neither resistance to a second treatment, nor changes in the expression of the anti-apoptotic protein Bcl-2 that contrasted with the overexpression of Bcl-2 observed in cells under doxorubicin treatment. In this regard, great efforts have been directed towards finding small molecules to inhibit these anti-apoptotic Bcl-2 family proteins to tackle anti-apoptotic adaptation of tumor cells [36].

Hence, targeting cell plasticity should provide a unique opportunity to improve the efficiency of existing therapies. The identification of signaling pathways involved in the cell phenotype changes during treatment may be a key to impede chemoresistance. The tandem constituted by CD24 and the MAPK p38 appears crucial in cell fate of MDA-MB-231 population. A preferential use of p38 in CD24⁺ cells have been noted under normal conditions which has been amplified under drug treatment in accord with the dynamics of CD24. The relationship between CD24 and p38 is supported by the incapability of SiCD24 cells to activate p38 under drug stress. Indeed, we hypothesize that the change in the dynamics of p38 activation that turned sustained early after CD24 translocation is linked to the increased surface CD24 expression observed in doxorubicin treated cells. In this regard, a very recent paper reported that MAPK cascade signaling dynamics (transient to sustained activation) may be controlled by the activation

kinetics of a given membrane receptor and not necessarily by the intracellular topology of the kinase networks. In the same study, it was proposed that redirecting signal dynamics may be a more fruitful and effective approach than controlling receptor activation in pathologic situation [37]. This is why we considered the profile of p38 activation as an important marker of the resistant cell identity (CD24⁺/DxR) and a choice target to impede phenotype switching during doxorubicin treatment.

The relevance of this strategy was verified when we compared the protein expression pattern of CD24⁺/DxR cells characterized by an overexpression of Bcl-2 and decline of cyclin D1 which are indicators of adaptative cellular reprogramming and also considered as markers of premature senescence [38–40] and naïve MDA-MB-231 cells treated with doxorubicin in the presence of the p38 inhibitor SB203580. In such conditions, SB203580 was capable not only to reduce Bcl-2 expression but to decrease the tri-methylation of H3K9 which constitutes a hallmark for stress response leading to cell reprogramming including drug resistance [41–43]. The benefit of this association was translated into a synergism of the cytotoxic effect of the combination of SB203580 and doxorubicin. The impact of SB203580 on cell fate may also correlate with the capacity of p38 to control expression of p53. Recently, p53 was also considered as a marker of cell reprogramming and acquisition of stemness [44]. Finally, an interesting point to note is that SB203580 had no effect on cell viability at 24h at the concentration used. This sustains the idea that p38 is an early active player in drug resistance by inducing cell identity changes, notably increasing cell survival marker expression.

To better assess the long term consequences of the rapid phenotype switching after drug treatment we have evaluated whether the slow-cycling state of CD24⁺/DxR cells was reversible after several weeks in drug-free medium since several studies have demonstrated the reversibility of senescence [45–48]. In fact, the revertant cells (named DxR/30), recovered their capacity to proliferate and importantly, have gained higher drug resistance and stronger migratory properties. This may be put in parallel with the constitutive activation of p38 in these cells. Interestingly, in the absence of the drug pressure and in proliferative conditions, the DxR/30 population retrieved a basal level of CD24⁺ cells. This follows mathematical models that tend to establish that tumor cell populations always maintain its heterogeneity at fixed ratio in dynamic conditions like proliferation [49].

So, here we have established a model to accompany the journey of MDA-MB-231 cells after drug treatment starting from their switching to CD24⁺ phenotype to their entry in slow-cycling state and reversion into proliferative cells. Notably, the sustained p38 phosphorylation observed in all the post-treatment stages may also indicate the participation of this MAPK in the network that successfully promotes the formation of metastasis at distant organs. Previous studies have shown that the program EMT (Epithelial-Mesenchymal Transition) has been involved in the distant metastases frequently detected following chemotherapy [50] that could also include p38. The monitoring of cell evolution throughout this journey has shown that they have conserved some features of previous state while having eliminated others, in other words, that they have acquired hybrid properties corresponding to a new identity.

The pertinence of using a drug pair, associating a leading drug with an inhibitor of cell reprogramming, was illustrated by the high efficiency of the combination of p38 inhibitor and doxorubicin on the killing of aggressive MDA-MB-231 breast cancer cell line as schematized in the figure 6.

Conclusions

This study reaffirms how a better understanding of the biology and molecular drivers of the CSCs plasticity will enable identification of new anticancer targets. More specifically, we have shown here that the cellular localization of CD24 associated with the changes in the dynamics of p38MAPK activation in breast tumor cells under drug stress plays a crucial role in the acquisition of drug resistance and new cell identity. The use of p38 inhibitor was able to disrupt this partnership and consequently to avoid chemotherapy-induced cell state transition. These results suggest that targeting p38 in aggressive breast cancer might be an interesting approach in order to overcome adaptive resistance to doxorubicin treatment.

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

The authors read and approved the final manuscript

Availability of supporting data

Not applicable

Competing interests

Not applicable

Funding

Not applicable

Conflict of Interest

The authors declare no conflicts of interest to disclose.

Contributions

HWH was involved in the laboratory experimental work, data analysis and interpretation and writing the manuscript. AMG participated in the study formulation. CR was involved in the supervision of the

experimental work, in the study formulation and writing the manuscript. The authors read and approved the final manuscript.

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Figures

Figure 1

Translocation of CD24 from cytosol to cell membrane is an early event in MDA-MB-231 cells under drug stress. (a) Evaluation of the localization of CD24 in MDA-MB-231 cell line. MDA-MB-231 cells were stained with anti-CD24/FITC (extracellular staining). Then cells were fixed, permeabilized and stained with anti-CD24/PE-Cy7 (intracellular staining). Pseudocolor plots are representative of triplicates. (b) Frequency of CD24+ cells according to the detection of CD24 in membrane (mb) or in cytosol (cyt). The

results are representative of four independent experiments (means $\pm$ SD of triplicates). (c) Visualization of CD24 localization by fluorescence microscopy. MDA-MB-231 cells were fixed, permeabilized and then stained using anti-CD24/PE-Cy7, anti-F-actin/Alexa488 and the nuclear dye DAPI. The yellow arrows indicate the membrane CD24 and the white arrows point to the cytosolic CD24. (d) CD24⁺ cell enrichment in MDA-MB-231 population during doxorubicin (Dx) treatment. MDA-MB-231 cells were treated with Dx (0.6 μ M) according to the indicated times followed or not by a drug-free medium (dfm) incubation. Then, an extracellular staining was performed using anti-CD24/FITC. The pseudocolor plots are representative of four independent experiments. (e) Histograms represent the shift of CD24 on x axis under different treatments. Table shows the MFI (median fluorescence intensity) values of surface CD24 expression calculated from figure b pseudocolor plots. Graph represents the means of triplicates obtained from the MFI calculation. (f) Reduction of CD24 translocation by brefeldin A in Dx-treated cells. MDA-MB-231 cells were treated with Dx(0.6 μ M) and/or with brefeldin A (1 μ g/ml) for 4h. When associated, brefeldin A was added 30 min before Dx. Then, an extracellular staining was performed using anti-CD24/PE-Cy7. The pseudocolor plots are representative of two independent experiments. (g) Effect of brefeldin A on CD24 localization in doxorubicin-treated cells visualized by fluorescence microscopy. MDA-MB-231 cells were treated with doxorubicin (0.6 μ M) and/or with brefeldin A (1 μ g/ml) by different times. Then, cells were stained using anti-CD24/Alexa488 and DAPI without fixation and permeabilization. DAPI was not used in the doxorubicin-treated groups. (h) Representative merged pseudocolor plots of CD24⁺ (red) and CD24⁻ (blue) subpopulations obtained from parental MDA-MB-231 cells after magnetic sorting. (i) Translocation of CD24 in CD24⁻ cells during doxorubicin treatment. CD24⁻ cells were treated with Dx (0.6 μ M) for 4h. Then cells were stained (extracellular staining) with anti-CD24/Pe-Cy7 antibody to evaluate the expression of surface CD24. The pseudocolor plots are representative of triplicates.

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Translocation of CD24 from cytosol to cell membrane is an early event in MDA-MB-231 cells under drug stress. (a) Evaluation of the localization of CD24 in MDA-MB-231 cell line. MDA-MB-231 cells were stained with anti-CD24/FITC (extracellular staining). Then cells were fixed, permeabilized and stained with anti-CD24/PE-Cy7 (intracellular staining). Pseudocolor plots are representative of triplicates. (b) Frequency of CD24+ cells according to the detection of CD24 in membrane (mb) or in cytosol (cyt). The

results are representative of four independent experiments (means $\pm$ SD of triplicates). (c) Visualization of CD24 localization by fluorescence microscopy. MDA-MB-231 cells were fixed, permeabilized and then stained using anti-CD24/PE-Cy7, anti-F-actin/Alexa488 and the nuclear dye DAPI. The yellow arrows indicate the membrane CD24 and the white arrows point to the cytosolic CD24. (d) CD24⁺ cell enrichment in MDA-MB-231 population during doxorubicin (Dx) treatment. MDA-MB-231 cells were treated with Dx (0.6 μ M) according to the indicated times followed or not by a drug-free medium (dfm) incubation. Then, an extracellular staining was performed using anti-CD24/FITC. The pseudocolor plots are representative of four independent experiments. (e) Histograms represent the shift of CD24 on x axis under different treatments. Table shows the MFI (median fluorescence intensity) values of surface CD24 expression calculated from figure b pseudocolor plots. Graph represents the means of triplicates obtained from the MFI calculation. (f) Reduction of CD24 translocation by brefeldin A in Dx-treated cells. MDA-MB-231 cells were treated with Dx(0.6 μ M) and/or with brefeldin A (1 μ g/ml) for 4h. When associated, brefeldin A was added 30 min before Dx. Then, an extracellular staining was performed using anti-CD24/PE-Cy7. The pseudocolor plots are representative of two independent experiments. (g) Effect of brefeldin A on CD24 localization in doxorubicin-treated cells visualized by fluorescence microscopy. MDA-MB-231 cells were treated with doxorubicin (0.6 μ M) and/or with brefeldin A (1 μ g/ml) by different times. Then, cells were stained using anti-CD24/Alexa488 and DAPI without fixation and permeabilization. DAPI was not used in the doxorubicin-treated groups. (h) Representative merged pseudocolor plots of CD24⁺ (red) and CD24⁻ (blue) subpopulations obtained from parental MDA-MB-231 cells after magnetic sorting. (i) Translocation of CD24 in CD24⁻ cells during doxorubicin treatment. CD24⁻ cells were treated with Dx (0.6 μ M) for 4h. Then cells were stained (extracellular staining) with anti-CD24/Pe-Cy7 antibody to evaluate the expression of surface CD24. The pseudocolor plots are representative of triplicates.

Figure 2

Translocation of CD24 from cytosol to membrane is observed in patient-derived breast cancer cells under drug stress. (a) Detection of CD24 in membrane and cytosol of breast cancer cell lines. MDA-MB-231, MACL-1 and MGSO-3 cells were stained with anti-CD24/Pe-Cy7 (extracellular staining). For intracellular staining, cells were permeabilized and stained with anti-CD24/PE-Cy7 (intracellular staining). Dot-plots are representative of triplicates. (b) Frequency of CD24+ cells according to the detection of CD24 in membrane (extracellular) or in cytosol (intracellular). (c) CD24+ cell enrichment in breast cancer cell lines during Dx treatment. MDA-MB-231, MACL-1 and MGSO-3 cells were treated with Dx (0.6 μ M) according to the indicated times. Then, an extracellular staining was performed using anti-CD24/FITC. The dot-plots are representative of triplicates. (d) Frequency of CD24+ cells under Dx treatment according to the detection of CD24 in membrane. Data were plotted as means of triplicates $\pm$ SD. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (One-way ANOVA with bonferroni post-test).

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Figure 3

Translocation of CD24 is associated with chemoresistance and overexpression of Bcl-2 (a) Schematic shows experimental design used to obtain cell populations for MTT (fig 3b) and western blot assays (fig 3c and 3d). dfm = drug-free medium; Dx = doxorubicin; CD24⁺/DxR = CD24⁺ doxorubicin-resistant cells. (b) CD24⁺/DxR cells become tolerant to a second Dx treatment. Parental MDA-MB-231 cells were cultured in the presence of Dx at 0.6 μ M for 48h. After obtention of CD24⁺/DxR cells, the medium was changed for

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Figure 4

Inhibition of p38 activity impedes phenotype switching of MDA-MB-231 cells under doxorubicin treatment. (a) Differential activation of MAPKs in CD24⁺ and CD24⁻ subpopulation. Parental and the magnetic sorted CD24⁺ and CD24⁻ cells were starved for 2h. Then, cells were submitted to time kinetic using fetal bovine serum (FBS) as stimulus. Cell lysates were immunoblotted with the depicted antibodies. The results are representative of two independent experiments. (b) Higher p38 phosphorylation (pp38)

MAPK in CD24+ cells. Parental MDA-MB-231 cells were stained with anti-CD24/Pe-Cy7 (extracellular staining) and anti-pp38/FITC (intracellular staining). The pseudocolor plots are representative of triplicates. (c) Decreased phosphorylation of p38 (pp38) in SiCD24 cells treated with Dx. SiC and SiCD24 cells were treated with Dx (0.6 μ M) for 24 hours. Cell lysates were immunoblotted with the depicted antibodies. All blots were performed at least twice. (d) Increased efficiency of Dx when associated with the p38 inhibitor SB203580. Cells were treated with SB203580 (SB) at 10 μ M or/and Dx at 0.6 μ M for 48h. Then, cells were counted and the growth percentage was calculated based on the initial number of cells before each treatment ($\% \text{ growth} = [\text{fn/in} - 1] \times 100$)#. Countings were performed in three independent experiments. (e) Reduction in the acquisition of Dx resistance in the presence of the p38 inhibitor SB203580. MDA-MB-231 cells were pre-treated with SB203580 (10 μ M) and/or doxorubicin (0.6 μ M) during 48h before the addition of Dx at different concentrations (varying between 0.07 μ M and 5 μ M) for 24h and then cell viability was measured by MTT assay. Curves were fitted with non-linear regression as means of triplicates. (f) Increased expression of Bax but decreased expression of Bcl2 and p53 after cell treatment with the association of the p38 inhibitor SB203580 and Dx. MDA-MB-231 cells were treated with SB203580 (10 μ M) and/or Dx (0.6 μ M) and cells were lysed after 48h treatment. Representative western blotting is shown and the data have been reproduced twice. (g) Representative light microscopy images of cell population at confluence (10⁶ cells) or subconfluence (5x10⁵ cells) density treated with SB203580 (10 μ M) and/or Dx (0.6 μ M) for 24h. Cell density and morphology were evaluated by microscopy (Evos Skedda). The microscopy images are representative of triplicates. (h) The p38 inhibitor SB203580 inhibits the tri-methylation state of H3K9 in Dx treated cells. After 24h of treatment with SB203580 (SB) at 10 μ M and/or Dx at 0.6 μ M, MDA-MB-231 cells were lysed and the status of histone 3 was analyzed by western blotting using antibodies against tri-methylated (H3K9me₃) or acetylated (H3K9ac) form of H3K9. (i) Schematic shows the histone modifications and its consequences on MDA-MB-231 cell phenotype after doxorubicin treatment. The reprogramming of doxorubicin-treated cells induced a cell cycle arrest and morphological modifications of MDA-MB-231 cells associated with the H3K9 histone state changes, acetylated vs methylated form under p38 control.

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Figure 4

Inhibition of p38 activity impedes phenotype switching of MDA-MB-231 cells under doxorubicin treatment. (a) Differential activation of MAPKs in CD24⁺ and CD24⁻ subpopulation. Parental and the magnetic sorted CD24⁺ and CD24⁻ cells were starved for 2h. Then, cells were submitted to time kinetic using fetal bovine serum (FBS) as stimulus. Cell lysates were immunoblotted with the depicted antibodies. The results are representative of two independent experiments. (b) Higher p38 phosphorylation (pp38)

MAPK in CD24+ cells. Parental MDA-MB-231 cells were stained with anti-CD24/Pe-Cy7 (extracellular staining) and anti-pp38/FITC (intracellular staining). The pseudocolor plots are representative of triplicates. (c) Decreased phosphorylation of p38 (pp38) in SiCD24 cells treated with Dx. SiC and SiCD24 cells were treated with Dx (0.6 μ M) for 24 hours. Cell lysates were immunoblotted with the depicted antibodies. All blots were performed at least twice. (d) Increased efficiency of Dx when associated with the p38 inhibitor SB203580. Cells were treated with SB203580 (SB) at 10 μ M or/and Dx at 0.6 μ M for 48h. Then, cells were counted and the growth percentage was calculated based on the initial number of cells before each treatment ($\% \text{ growth} = [\text{fn/in} - 1] \times 100$)#. Countings were performed in three independent experiments. (e) Reduction in the acquisition of Dx resistance in the presence of the p38 inhibitor SB203580. MDA-MB-231 cells were pre-treated with SB203580 (10 μ M) and/or doxorubicin (0.6 μ M) during 48h before the addition of Dx at different concentrations (varying between 0.07 μ M and 5 μ M) for 24h and then cell viability was measured by MTT assay. Curves were fitted with non-linear regression as means of triplicates. (f) Increased expression of Bax but decreased expression of Bcl2 and p53 after cell treatment with the association of the p38 inhibitor SB203580 and Dx. MDA-MB-231 cells were treated with SB203580 (10 μ M) and/or Dx (0.6 μ M) and cells were lysed after 48h treatment. Representative western blotting is shown and the data have been reproduced twice. (g) Representative light microscopy images of cell population at confluence (10⁶ cells) or subconfluence (5x10⁵ cells) density treated with SB203580 (10 μ M) and/or Dx (0.6 μ M) for 24h. Cell density and morphology were evaluated by microscopy (Evos Skedda). The microscopy images are representative of triplicates. (h) The p38 inhibitor SB203580 inhibits the tri-methylation state of H3K9 in Dx treated cells. After 24h of treatment with SB203580 (SB) at 10 μ M and/or Dx at 0.6 μ M, MDA-MB-231 cells were lysed and the status of histone 3 was analyzed by western blotting using antibodies against tri-methylated (H3K9me₃) or acetylated (H3K9ac) form of H3K9. (i) Schematic shows the histone modifications and its consequences on MDA-MB-231 cell phenotype after doxorubicin treatment. The reprogramming of doxorubicin-treated cells induced a cell cycle arrest and morphological modifications of MDA-MB-231 cells associated with the H3K9 histone state changes, acetylated vs methylated form under p38 control.

Figure 5

CD24+/DxR cells become proliferative and more invasive than naïve cells after a long-lasting period in drug-free environment. (a) Schematic shows the journey of CD24+/DxR cells from their switching to CD24+ phenotype to their entry in slow- cycling state and reversion into proliferative cells. CD24+/DxR cells were exposed to a stress condition (fetal bovine serum starvation = 1% FBS) during 48h. (b) Differences in morphology between naïve MDA-MB-231 and CD24+/DxR cells. Naïve MDA-MB-231 and

CD24+/DxR cells were fixed, permeabilized and stained using anti-F-actin/Alexa488 and the nuclear dye DAPI. DAPI was not used in CD24+/DxR group due to co-localization with doxorubicin. (c) CD24+/DxR cells retrieve their capacity to proliferate (named as DxR/30 or revertant). Naive MDA-MB-231 and DxR/30 cells were incubated with 10 μ M of BrdU (thymine analogue base) for 4h. Then, cells were stained with anti-BrdU/PerCP-Cy5.5 and anti- γ H2AX/Alexa647 (intracellular staining). The low and high proliferative subpopulations were defined and analyzed. The pseudocolor plots are representative of triplicates. (d) DxR/30 cell population returns the basal ratio of CD24+ cells. Cells were stained with the fluorescent antibody anti-CD24/Pe-Cy7 (extracellular staining). Pseudocolor plots are representative of triplicates. (e) Constitutive activation of p38 MAPK in DxR/30 cells. After starvation for 2h, cells were stimulated with serum in different times. Cell lysates were immunoblotted with the depicted antibodies. All blots were performed at least twice. (f) Higher resistance of DxR/30 cells to Dx treatment. MDA-MB-231 and DxR/30 cells were treated with Dx (0.6 μ M) for 24h. Then cells were counted, re-seeded in the same density and cultured in normal conditions (DMEM 10% FBS). Cell density and morphology were analyzed by light microscopy (Evos Skedda) at 5 and 12 days post-doxorubicin treatment. (g) DxR/30 cells are more invasive than MDA-MB-231 cells. MDA-MB-231 and DxR/30 cells were seeded (8x10⁵) in 6 wells plate and a scratch performed according to Mat and Med. Cell migrations were registered by light microscopy (Evos Skedda) at 24, 48 and 72 hours post scratch. Using ImageJ software the wound healing was measured and a closing-time percentage was calculated based on the initial scratch size of each cell type. Curves were fitted as means of duplicates +/- SD. ** p<0,01 (Two-way ANOVA with Bonferroni post-test).

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Figure 6

Schematic shows breast cancer cell population phenotype changes under drug stress toward a drug tolerant state arising through translocation of CD24 and sustained p38 activation. (a) In proliferative conditions, CD24 is mainly detected in the cytosol of all cells and the population is characterized by a low frequency of CD24+ cells (cells carrying CD24 in membrane). The transient phosphorylation of p38 leads to cyclin D1 expression and cell cycle progression. (b) Under drug treatment, CD24 translocation associated with an increased CD24 density on cell membrane occurs in every given cell. In accord with this, a sustained and strong p38 activation, an increased Bcl-2 expression and a tri-methylation of H3K9 are observed indicating a phenotype switching. (c) This transition state is sensitive to p38 inhibition since

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