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Dynamic emergence of the mesenchymal CD44^{pos}CD24$^{neg/low}$ phenotype in *HER2*-gene amplified breast cancer cells with *de novo* resistance to trastuzumab (Herceptin)

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ABSTRACT

Evidence is mounting that the occurrence of the CD44^{pos}/CD24$^{neg/low}$ cell population, which contains potential breast cancer (BC) stem cells, could explain BC clinical resistance to HER2-targeted therapies. We investigated whether *de novo* refractoriness to the anti-HER2 monoclonal antibody trastuzumab (Tzb; Herceptin) may relate to the dynamic regulation of the mesenchymal CD44^{pos}/CD24$^{neg/low}$ phenotype in HER2-positive BC. We observed that the subpopulation of Tzb-refractory JIMT-1 BC cells exhibiting CD44^{pos}/CD24$^{neg/low}$-surface markers switched with time. Low-passage JIMT-1 cell cultures were found to spontaneously contain ~10% of cells bearing the CD44^{pos}/CD24$^{neg/low}$ immunophenotype. Late-passage (>60) JIMT-1 cultures accumulated ~80% of CD44^{pos}/CD24$^{neg/low}$ cells and closely resembled the CD44^{pos}/CD24$^{neg/low}$-enriched (~85%) cell population constitutively occurring in HER2-negative MDA-MB-231 mesenchymal BC cells. Dynamic expression of mesenchymal markers was not limited to CD44/CD24 because high-passages of JIMT-1 cells exhibited also reduced expression of the HER2 protein and over-secretion of pro-invasive/metastatic chemokines and metalloproteases. Accordingly, late-passage JIMT-1 cells displayed an exacerbated migratogenic phenotype in plastic, collagen, and fibronectin substrates. Intrinsic genetic plasticity to efficiently drive the emergence of the CD44^{pos}/CD24$^{neg/low}$ mesenchymal phenotype may account for *de novo* resistance to HER2 targeting therapies in basal-like BC carrying *HER2* gene amplification.

1. Introduction

Numerous reports have clinically substantiated the notion that *HER2* overexpression of HER2 (*erb*B2; Her-2/*neu*) oncogene is associated with unfavorable prognosis, shorter relapse time, resistance to traditional systemic therapy, and decreased overall survival in breast cancer (BC) patients [1–4]. This has led to the development of trastuzumab (Tzb; Herceptin), a recombinant humanized monoclonal antibody against the extracellular domain of HER2 receptor [5–7]. Pivotal trials showing clinical benefit of Tzb in combination with chemotherapy have led to a new standard of care for women with *HER2* + metastatic and early-stage BC [8–12]. Unfortunately, most women with *HER2* + metastatic BC who respond initially to Tzb develop acquired resistance within months or years [13–20]. Indeed, 70% of HER2-overexpressing metastatic BC shows primary resistance to Tzb as a single agent and approximately 15% of women diagnosed with early *HER2* + disease are *de novo* resistant to Tzb and relapse in spite of treatment with Tzb-based therapies [21,22].

Intrinsic Tzb resistance in a cell line isolated from the pleural fluid of a *HER2* + BC patient with progressive disease on Tzb (i.e., JIMT-1) constitutes an excellent scenario to discover molecular explanations for *de novo* Tzb resistance [23–25]. High-resolution genomic profiles have confirmed that, while Tzb-sensitive *HER2* gene-amplified BT-474 and SKBR3 BC cell lines rather displayed a luminal B-like gene expression phenotype, JIMT-1 was the only cell line that had closest resemblance to the *HER2* + gene expression BC subtype [26]. JIMT-1 cells express both basal CK5/14 and luminal CK8/18 cytokeratins [23], which may reflect the "stem/progenitor cell" origin of JIMT-1. In this regard, CD44 – a transmembrane receptor that binds to hyaluronan in the extracellular matrix to

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induce cystoskeletal rearrangements facilitating adhesion and invasion – is up-regulated also in JIMT-1 cells [25]. Because CD44 positivity has been shown to be a cardinal feature of BC stem cells [27] and because Tzb treatment fails to decrease the fraction of JIMT-1 cells positive for the stem cell marker aldehyde dehydrogenase (ALDH1) [28,29], it is reasonable to suggest that BC stem cell-like phenotypes may be at the root of resistance of Tzb in this cell line [30].

Recent evidence indicates that the success of HER2-targeted therapies including Tzb may be explained, at least in part, by their direct activity against HER2 + BC stem cells [28,31,32]. Intriguingly, a variety of possible mechanisms of escape from Tzb appears to involve many of the same molecular markers that have been implicated in the biology of BC stem cells [30]. However, there are few direct links between BC stem cells and the emergence of resistance to Tzb therapy. Here, we sought to establish if *ab initio* responses to Tzb might correlate with the presence of the BC initiating $CD44^{pos}/CD24^{neg/low}$ mesenchymal phenotype in *HER2* gene-amplified BC.

2. Materials and methods

2.1. Culture conditions

MCF-7, SKBR3 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and they where grown in Improved MEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine. JIMT-1 cells were obtained from the German Collection of Microorganisms and they were grown in F-12/DMEM (1:1) supplemented with 10% FBS and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. Flow cytometry

Cell surface expression of CD24 and CD44 markers was analyzed on a BD FacScalibur using combinations of fluorochrome-conjugated monoclonal antibodies obtained from BD Biosciences (San Diego, CA, USA) against human CD44 (FITC; cat.#555478) and CD24 (PE; cat.#555428) or their respective isotype controls as described elsewhere [33–37].

2.3. HER2-specific ELISA

Determination of HER2 protein content was carried out with a commercially available quantitative ELISA (Human neu Quantitative ELISA System; Oncogene Science, Cambridge, MA, USA) and according to the manufacturer's protocol.

2.4. Immunofluorescence microscopy

Sub-cellular expression of CD44, CD24 and HER2 was monitored using the automated confocal imaging platform BD Pathway™ 855 Bioimager System (Becton Dickinson Biosciences, San Jose, CA, USA) as described elsewhere [17]. Both acquisition and merging of immunofluorescence images were carried out following BD Biosciences protocols and according to the Recommended Assay Procedure using BDAttovision™ software.

2.5. Cell viability

Cell viability effects upon exposure to Tzb were analyzed in a tetrazolium-based colorimetric (MTT) assay [17].

2.6. Cell migration assays

Cell migration activity of SKBR3 and JIMT-1 cells was monitored using the Oris™ Cell Migration Assay-TriCoated kit from Platypus Technologies and according to the manufacturer's protocol.

2.7. Antibody-based arraying of cytokines and metalloproteases

Assays for cytokine and metalloprotease profiling were carried out as per manufacturer's instructions (RayBiotech, Inc. Norcross, GA, USA) using RayBio® Human Cytokine Array 3 and RayBio® Human Matrix Metalloproteinase Antibody Array 1 membranes.

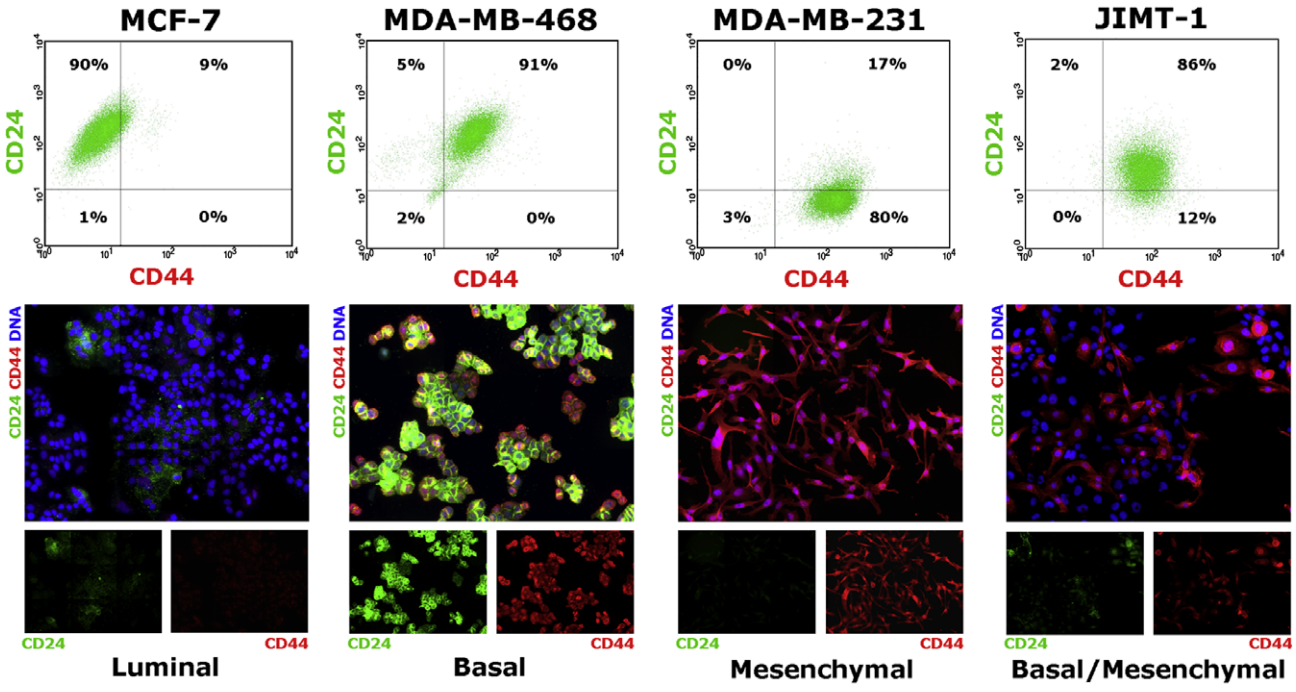

Fig. 1. Progenitor cell properties ($CD44^{pos}/CD24^{neg/low}$) of various breast cancer cell lines as assessed by flow cytometry (*top*) and immunofluorescence microscopy (*bottom*).

2.8. Statistics

Two-group comparisons were performed by the Student́s t test for paired and unpaired values. Comparisons of means of ⩾ 3 groups were performed by ANOVA.

3. Results

3.1. Tzb-refractory JIMT-1 cells differ to other breast cancer cell lines in their distribution of CD44 and CD-negative and vimentin-positive, possessed a sign24 cell surface markers

Fig. 1 (*top panels*)shows a summarized analysis of MCF-7, MDA-MB-468, MDA-MB-231 and JIMT-1 BC cells with respect to four cell population fractions defined in terms of CD44 and CD24 cell surface markers. MCF-7 cells were mainly $CD24^{pos}/CD44^{neg/low}$ (90%), which was consistent with their luminal-type classification. Highly metastatic (but not invasive) MDA-MB-468 basal cells, which are ERα-negative and vimentin-negative, likewise lacked a $CD24^{pos}/CD44^{neg/low}$ population and were largely positive for both CD24 and CD44 (91%). Highly metastatic (and invasive) MDA-MB-231 mesenchymal cells, which are ERα-negative and vimentin-positive, possessed a significantly increased $CD44^{pos}/CD24^{neg/low}$ population (80%). A low passage pleural effusion explant of Tzb-refractory JIMT-1 cells, which are ERα-negative and vimentin-positive (data not shown), were highly enriched with the $CD44^{pos}/CD24^{pos}$ fraction (86%) and spontaneously contained ∼12% of cells exhibiting the $CD44^{pos}/CD24^{neg/low}$ mesenchymal phenotype [38,39]. Indirect immunofluorescence imaging of CD24 and CD44 confirmed that an intermediate basal/mesenchymal rather than luminal phenotype closely correlated with the content of $CD24^{pos}$ and $CD44^{pos}$ cells in Tzb-refractory JIMT-1 cells (Fig. 1, *bottom panels*).

3.2. Tzb-refractory JIMT-1 cell cultures spontaneously evolve to $CD44^{pos}/CD24^{neg/low}$-enriched mesenchymal-like BC phenotypes

We sought to determine whether the $CD44^{pos}/CD24^{neg/low}$ mesenchymal population was maintained with increasing JIMT-1 cell passage. Flow cytometry analyses revealed that Tzb-sensitive SKBR3 cells lacked a $CD44^{pos}/CD24^{neg/low}$ subpopulation and maintained their $CD24^{pos}/CD44^{neg/low}$–enriched (∼90%) luminal phenotype after continuous subculture (Fig. 2, *top panels*). Conversely, the subpopulation of JIMT-1 cells exhibiting $CD44^{pos}/CD24^{neg/low}$-surface mesenchymal immunophenotype switched with time (Fig. 2, *bottom panels*). An obvious subpopulation of $CD44^{pos}/CD24^{neg/low}$ cells (∼50%) was found to occur after 15–20 passages (with the first culture after obtaining JIMT-1 cells as passage 4). At late passages (>60), >80% of JIMT-1 became $CD44^{pos}/CD24^{neg/low}$ and almost reached $CD44^{pos}/CD24^{neg/low}$ (∼85%) cell numbers constitutively found in *HER2*-negative MDA-MB-231 mesenchymal BC cells. Indirect immunofluorescence imaging of CD44 and CD24 markers in late-passage JIMT-1 cells confirmed a significant enrichment in the number of cells exhibiting high levels of CD44 expression (Fig. 2, *bottom*).

3.3. HER2 protein expression is downregulated in high-passage JIMT-1 cells

Indirect immunofluorescence assays revealed that cell membrane-associated HER2 was significantly reduced in mesenchymal-like high-passage JIMT-1 cultures when compared to low-passage JIMT-1 cultures (Fig. 3, *left panels*). Quantitative assessment of

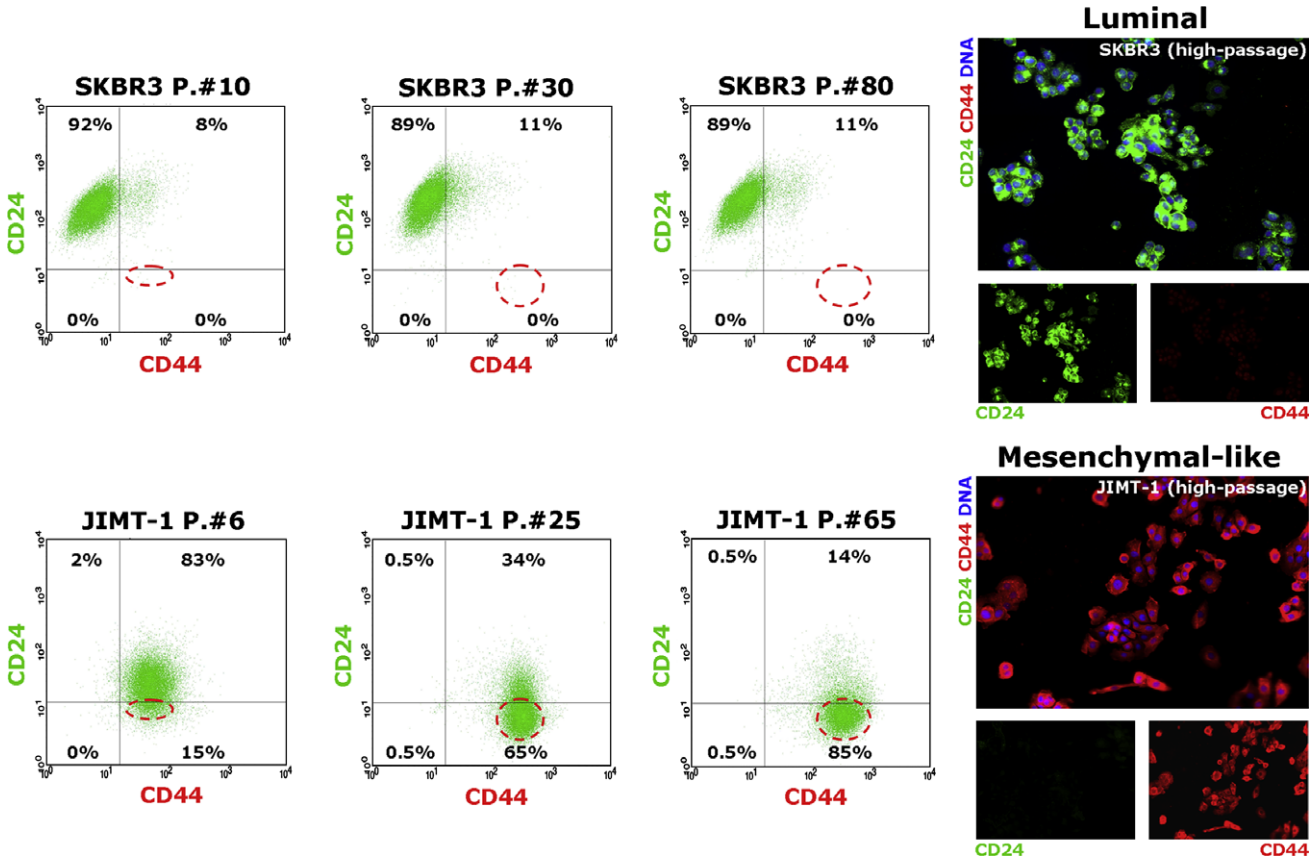

Fig. 2. Dynamic emergence of the stem/progenitor $CD44^{pos}/CD24^{neg/low}$ phenotype in Tzb-refractory JIMT-1 cells as assessed by flow cytometry (*left*) and immunofluorescence microscopy (*right*).

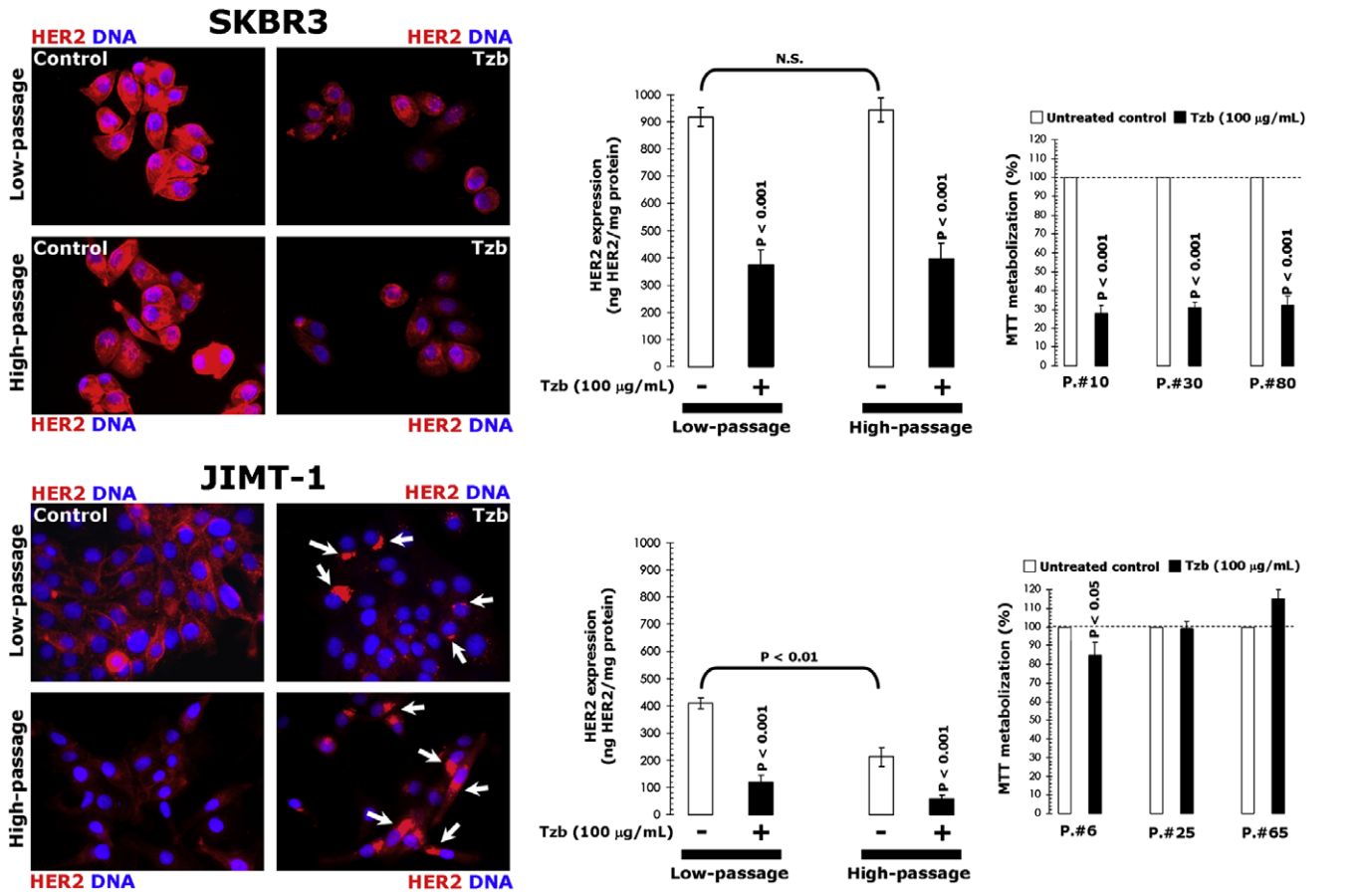

Fig. 3. Dynamic down-regulation of HER2 protein expression in Tzb-refractory JIMT-1 cells. *Left panels.* Sub-cellular distribution of HER2 protein as assessed by immunofluorescence microscopy; *Middle panels.* Quantitative assessment of HER2 protein expression by HER2 microtiter ELISA; *Right panels.* Metabolic status as evaluated by MTT-based cell viability assays.

HER2 protein expression in whole cell lysates obtained from early and late JIMT-1 cell passages confirmed that HER2 downregulation (~50% reduction) paralleled enrichment in the $CD44^{pos}/CD24^{neg/low}$ population (Fig. 3, *middle panels*). SKBR3 cells likewise exhibited unaltered expression of HER2 protein in early- and late-passages.

Tzb treatment efficiently promoted loss of cell membrane-associated HER2 and drastically increased cytosolic trafficking of vesicular-associated HER2 in the peri-nuclear region of early- and late-passages of JIMT-1 cells (Fig. 3, *left panels*). Although we failed to detect an equivalent trafficking of HER2 in Tzb-sensitive SKBR3 cells, quantitative measurements of HER2 protein in whole cell lysates confirmed that Tzb exposure (48 h) reduced HER2 expression in JIMT-1 as efficiently as in Tzb-sensitive SKBR3 cells (Fig. 3, *middle panels*). Tzb-induced HER2 downregulation in JIMT-1 and SKBR3 cells did not result into equivalent reductions in JIMT-1 and SKBR3 cell viability. Early- and late-passages of SKBR3 cells displayed an exquisite sensitivity to the growth inhibitory effects of Tzb. Cell viability of low-passage JIMT-1 cells was slightly decreased following exposure to 100 μg/mL Tzb. MTT reduction (and, hence, energetic metabolism) was rather enhanced when high-passages of JIMT-1 cells were challenged with Tzb. (Fig. 3, *right panels*).

3.4. High-passage JIMT-1 cells exhibit a highly-migratogenic phenotype and over-secrete pro-invasive/metastatic proteins

Once we confirmed that low-passage JIMT-1 cells were poorly migratogenic [38,39], we sought to establish whether enrichment with $CD44^{pos}/CD24^{neg/low}$ mesenchymal cells related to changes in the cell migratory behavior of JIMT-1 cultures. $CD44^{pos}/CD24^{neg/low}$-negative SKBR3 cells likewise exhibited a very low locomotory behavior when compared to the highly migratogenic $CD44^{pos}/CD24^{neg/low}$-enriched JIMT-1 cells. Tzb treatment failed to block high locomotory activity of JIMT-1 cells irrespective of the plastic, collagen, and fibronectin extracellular matrix (ECM) substrates (Fig. 4, *left panels*).

We finally investigated the relationship between the $CD44^{pos}/CD24^{neg/low}$ mesenchymal immunophenotype in high-passages of JIMT-1 cells and the expression of proteins implicated in invasion/metastasis (Fig. 4, *right panels*). Cytokine antibody-based arrays revealed that JIMT-1 cells secreted enormous amounts of interleukin-6 (IL-6) and IL-8 when compared to SKBR3 cells. JIMT-1 cells secreted slightly higher amounts of GROα (growth-related oncogene alpha), IL-7, M-CSF (Macrophage-colony stimulating factor), MDC (macrophage-derived chemokine; CCL22), and MIG (Monokine induced by Interferon-Gamma). JIMT-1 cells exhibited decreased secretion of I-309 (Inflammatory cytokine-309; CCL1), MCP-1 (Monocyte chemoattractant protein-1; CCL2), and RANTES (CCL5) when compared to SKBR3 cells. MMPs antibody-based arrays revealed that JIMT-1 drastically up-regulated the secretion of metalloproteinase-1 (MMP-1; interstitial collagenase) and metalloproteinase-3 (MMP-3; stromelysin-1). JIMT-1 conditioned media exhibited a significant increase of the tissue inhibitors of MMP-1 (TIMP-1) and MMP-2 (TIMP-2).

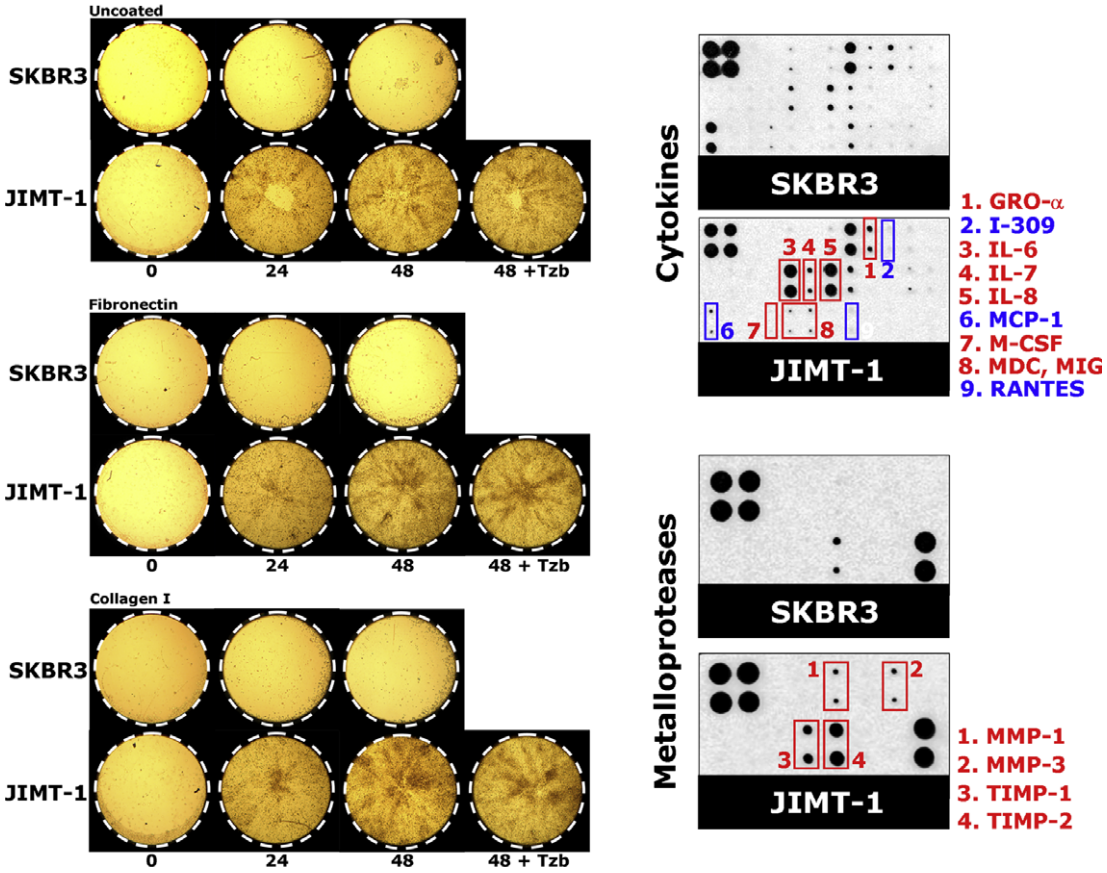

Fig. 4. Tzb-refractory JIMT-1 cells exhibit pro-invasive properties. *Left panels.* Representative analysis of cell migration assays using the Oris™ Cell Migration Assay-TriCoated kit. *Right panels.* Representative cytokine and MMP profiles using antibody-based microarrays. *Red:* Up-regulation; *blue:* Down-regulation.

4. Discussion

We have explored whether *de novo* resistance to Tzb in *HER2* gene-amplified JIMT-1 BC cells with can be explained within the framework of the cancer stem-cell hypothesis [30,40–43]. The cell surface phenotype $CD44^{pos}/CD24^{neg/low}$ initially described as a feature of BC stem cells has been associated with the expression of basal/mesenchymal or myoepithelial markers in BC cell lines [27]. While the $CD44^{pos}/CD24^{neg/low}$ phenotype is enriched in basal-like and *BRCA1* mutant BC [34–36], BCs displaying *HER2* gene overexpression are predominantly positive for CD24 [34], a negative regulator of the pro-metastatic chemokine receptor CXCR4 [44]. Because the CD24 antigen is highly expressed in luminal cells and because HER2 expression could not be detected in CD44-positive cells isolated from normal breast tissue, it has been suggested that HER2 overexpression may be a relatively late event in HER2-positive BC tumorigenesis as *HER2*-positive BC mainly originate from luminal cells [34]. It is well recognized, however, that HER2 overexpression frequently occurs in premalignant lesions, such as DCIS [45]. Moreover, HER2 overexpression significantly increases the fraction of BC cells positive for ALDH1 [28], a marker of normal and cancerous human mammary epithelial cells with stem/progenitor properties [29]. The fact that Alexe et al. reported two distinct gene expression profiles for HER2-positive tumors that markedly differed in their long-term outcomes may reconcile these discrepancies regarding the ultimate origin of HER2-positive BC [46]. Our current findings provide a dynamic perspective not only to the heterogeneous nature of the initiating pathways that originate HER2-positive BC but also to the role of $CD44^{pos}/CD24^{neg/low}$ mesenchymal cells in the intrinsic refractoriness of some HER2 + BC subgroups to Tzb.

Low-passage JIMT-1 metastatic pleural effusion cells possessed an intermediate basal/mesenchymal immunophenotype highly enriched in the $CD44^{pos}/CD24^{pos}$ fraction. Interestingly, JIMT-1 cell cultures suffered a spontaneous enrichment in cells bearing the $CD44^{pos}/CD24^{neg/low}$ mesenchymal immunophenotype after multiple passages. $CD44^{pos}/CD24^{neg/low}$-enriched JIMT-1 cell cultures over-secreted several MMPs, TIMPs and interleukins such as IL-6 and IL-8, a unique secretome that is expected in highly-migratogenic & -metastatic BC cells belonging to the basal/mesenchymal or the myoepithelial group [33]. Intrinsic plasticity of JIMT-1 cells for spontaneous transition to highly motile/invasive mesenchymal-like phenotypes was accompanied by significant reductions in whole and cell membrane-associated HER2 oncoprotein levels. In this regard, the induction of epithelial-mesenchymal transition (EMT) –a highly conserved cellular program allowing conversion of differentiated epithelial cells to motile mesenchymal cells and to generate cells with stem-like features such as the $CD44^{pos}/CD24^{neg/low}$ phenotype [47,48]- has been recently associated with progressive loss of HER2 expression at the cell membrane in *HER2*-positive BC cells [49]. The notion that EMT-mediated loss of HER2 might be involved in Tzb resistance was partially supported by the fact that, in the presence of *HER2* gene amplification in both epithelial and mesenchymal of BC tissues, immunohistochemical staining of membrane HER2 was lost in the mesenchymal areas [49]. In this scenario, our current description of the presence of a putative "tumorigenic" signature in JIMT-1 cells –as defined using relative content of $CD44^{pos}/CD24^{neg/low}$ mesenchymal cells- strongly suggests that *HER2* gene-amplified BC cells intrinsically resistant to HER targeting therapies may arise from very early breast epithelial precursors enriched in EMT and/or stem cell-like features.

5. Conclusions

Spontaneous morphologic and phenotypic EMT-like changes that have been recognized to occur within mostly HER2-negative genetic contexts (e.g. basal-like BC [50]) can take place also in *HER2* gene-amplified scenarios, thus allowing a dynamic emergence of biologically aggressive $CD44^{pos}/CD24^{neg/low}$ mesenchymal progenies intrinsically refractory to HER2-targeted therapies such as Tzb.

Conflicts of Interest

The authors declare they have no conflict of interest.

Acknowledgments

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