SPANDIDOS PUBLICATIONS

The anti-diabetic drug metformin suppresses the metastasis-associated protein CD24 in MDA-MB-468 triple-negative breast cancer cells

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Abstract. CD24, a mucin-like adhesion molecule that enhances the metastatic potential of malignant cells, has been suggested to be a marker of poor prognosis in breast carcinomas. The tumor-initiating potential of $CD44^{pos}CD24^{pos}$ cell populations has been recently recognized and, accordingly, distant metastases are largely composed of CD24-positive cells in breast cancer patients refractory to treatment. Therefore, new therapeutic strategies aimed at down-regulating CD24 may negatively regulate the dissemination of tumor cells and formation of metastasis. Here, we reveal that suppression of CD24 protein expression is a crucial event in the molecular mechanisms underlying the growth-inhibitory effects of the anti-diabetic drug metformin in MDA-MB-468 triple-negative (basal-like) breast cancer cells. First, we confirmed that, among the different molecular classes of breast cancer, basal-like breast cancer cells were significantly more sensitive to the growth-inhibitory effects of metformin. Second, we observed a positive correlation between the growth inhibitory activity of metformin and the relative enrichment in cells bearing the $CD44^{pos}CD24^{pos}$ immunophenotype. Third, high-content indirect immunofluorescence imaging assays revealed that CD24 protein levels were drastically decreased in the presence of growth-inhibitory concentrations of metformin. Fourth, to preliminary assess the clinical relevance of metformin's anti-CD24 effects we took advantage of the recently developed ROCK online interface (http://rock.icr.ac.uk/), a publicly accessible portal that allows rapid integration of breast cancer functional and molecular profiling datasets. When we evaluated the impact of CD24 expression on distant metastasis-free survival (DMFS) in microarray gene expression breast cancer datasets, Kaplan-Meier survival analyses and log-rank tests comparing DMSF for CD24-high and CD24-low breast carcinomas revealed that patients with CD24-high tumors tended to have a shorter DMFS. These findings, altogether, suggest that the ability of metformin to suppress the oncogene, metastasis promoter and breast cancer stem cell marker CD24 may open a novel molecular avenue in the therapeutic management of highly-metastastic subgroups of triple-negative (basal-like) breast cancers naturally enriched with $CD44^{pos}CD24^{pos}$ tumor-initiating cell populations.

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Introduction

Contrary to current '$CD44^{pos}CD24^{neg/low}$ centric' thought regarding the developmental plasticity of tumor-initiating mammary epithelial cells (1,2), recent studies have revealed that not only $CD44^{pos}CD24^{neg/low}$ cells can give rise to $CD44^{pos}CD24^{pos}$ cells, as expected for a cancer stem cell (3), but that the converse can also occur; $CD44^{pos}CD24^{pos}$ cells can give rise to their $CD44^{pos}CD24^{neg/low}$ counterparts and single cells from either phenotype are capable of initiating tumors as xenografts with high efficiency (4). This previously unrecognized developmental and tumor-initiating potential of $CD44^{pos}CD24^{pos}$ cells implies that current efforts to develop agents that specifically target the $CD44^{pos}CD24^{neg/low}$ population may be destined to fail unless the potential of either $CD44^{pos}$ population to regenerate the other is also prevented (4,5). Although the lack of CD24 has been highlighted within the putative $CD44^{pos}CD24^{neg/low}$ breast cancer stem cells, new therapeutic strategies aimed to down-regulate CD24, a mucin-like adhesion molecule that enhances the metastatic potential of malignant cells by acting as a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets, may negatively regulate the dissemination of tumor cells and formation of metastasis through by preventing activation of

the CD24/P-selectin pathway (6-8). In this regard, the demonstration that distant metastases are largely composed of $CD24^{pos}$ cells in patients refractory to treatment not only highlights the fact that non-$CD44^{pos}CD24^{neg/low}$ breast cancer stem cells can kill the patients but also supports the notion that differentiated $CD24^{pos}$ cells can become more invasive and initiate metastasis themselves (3,9,10). Given that higher levels of CD24 correlated with estrogen receptor-α (ERα) negativity and estrogen exposure efficiently down-regulates CD24 (11-13), anti-CD24 strategies might be particularly important in breast carcinomas lacking or losing ER as these ER-negative tumors cannot longer repress CD24 expression and therefore may more readily engage the CD24/P-selectin and metastasis pathway.

It has been recently reported that the anti-diabetic biguanide metformin, which significantly reduces cancer incidence and improves cancer patients' survival in type 2 diabetics (reviewed in refs. 14 and 15), exhibits unique biological and molecular effects against basal-like (triple-negative) breast cancer cells (16-18). In this regard, we recently sought to investigate whether the exacerbated response of highly-metastatic basal-like breast cancer cells to metformin might relate to metformin's ability to regulate CD24 expression and, hence, the metastatic potential of $CD44^{pos}CD24^{pos}$ cell populations. By employing $CD44^{pos}CD24^{pos}$-enriched MDA-MB-468 triple-negative breast cancer cells we reveal for the first time that suppression of CD24 protein expression is a crucial event in the molecular mechanisms underlying the growth-inhibitory effects of the anti-diabetic drug metformin in triple-negative (basal-like) breast cancer cells. In addition, we illustrate a previously unrecognized relationship between the expression levels of CD24 in primary tumors and the duration of distant metastasis-free survival (DMSF).

Materials and methods

Human breast cancer cell lines and culture conditions. MCF-7, MDA-MB-231 and MDA-MB-468 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and they where routinely grown in Improved MEM (IMEM; BioSource International; Invitrogen S.A., Barcelona, Spain) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine. JIMT-1 human breast cancer cell line was established at Tampere University and is available from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/). JIMT-1 cells 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. Cells were screened periodically for *Mycoplasma* contamination.

Metabolic status assessment (MTT-based cell viability assays). Cells were seeded at a density of ~3000 cells per well in a 96-well plate. The next day, cells were treated with graded concentrations of metformin. After 5 days of treatment (metformin was not renewed during the entire period of culture treatment), cells were incubated with a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO, USA) at a concentration of 5 mg/ml for 3 h at 37˚C. The supernatants were then carefully aspirated, 100 µl of DMSO were added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were read at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). Cell viability effects upon exposure to metformin were analyzed as percentages of the absorbance obtained in untreated control cells. For each treatment, cell viability was evaluated as a percentage using the following equation: (A_{570} of treated sample/A_{570} of untreated sample) x 100. Breast cancer cell sensitivity to metformin was expressed in terms of the concentration of the drug required to decrease by 50% cell viability (IC_{50} value). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} value was defined as the concentration of metformin that produced 50% reduction in control absorbance (by interpolation).

Flow cytometry. Cell cultures growing in regular medium (or supplemented with metformin as specified), were washed once with phosphate-buffered saline (PBS) and then harvested with 0.05% trypsin/0.025% EDTA into single cell suspensions. Detached cells were washed with PBS containing 1% FBS and 1% penicillin/streptomycin (wash buffer), counted and resuspended in the wash buffer (10^6 cells/100 µl). Combinations of fluorochrome-conjugated monoclonal antibodies obtained from BD Biosciences (San Diego, CA, USA) against human CD44 (FITC; cat. no.555478) and CD24 (PE; cat. no. 555428) or their respective isotype controls were added to the cell suspension at concentrations recommended by the manufacturer and incubated at 4˚C in the dark for 30-40 min. Labelled cells were washed in the wash buffer to eliminate unbound antibody, then fixed in PBS containing 1% paraformaldehyde, and then analyzed no longer than 1 h post-staining on a BD FACScalibur (BD Biosciences).

Immunofluorescence staining and high-content confocal imaging. Cells were seeded at ~5000 cells/well in 96-well clear bottom imaging tissue culture plates (Becton-Dickinson Biosciences, San Jose, CA, USA) optimized for automated imaging applications. Triton® X-100 permeabilization and blocking, primary antibody staining (1:50 dilution), secondary antibody staining using Alexa Fluor® 488/594 goat anti-rabbit/mouse IgGs (Invitrogen, Molecular Probes, Eugene, OR, USA) and counterstaining (using Hoechst 33258; Invitrogen) were performed following BD Biosciences protocols. Images were captured in different channels for Alexa Fluor® 488 (pseudo-colored green), Alexa Fluor® 594 (pseudo-colored red) and Hoechst 33258 (pseudo-colored blue) on a BD Pathway™ 855 Bioimager System (Becton-Dickinson Biosciences) with x20 or x40 objectives (NA 075 Olympus). Both acquisition and merging of images were carried out according to the Recommended Assay Procedure using BDAttovision™ software.

Kaplan-Meier survival curves. We investigated the prognostic significance of CD24 on distant metastasis-free survival (DMSF) using a recently developed breast cancer functional genomics resource (ROCK; rock.icr.ac.uk), a publicly accessible online interface for the integration of breast cancer functional and molecular profiling datasets (19).

CELL LINE	$CD44^{neg}CD24^{neg}$	$CD44^{neg}CD24^{pos}$	$CD44^{pos}CD24^{pos}$	$CD44^{pos}CD24^{neg}$	Metformin IC_{50}
1. MCF-7	1	90 ± 4	9 ± 2	0	18 ± 2
2. MDA-MB-231	3	0	17 ± 2	80 ± 3	15 ± 2
3. JIMT-1	1	1	34 ± 4	63 ± 5	12 ± 2
4. MDA-MB-468	0	2	86 ± 3	12 ± 2	3 ± 1

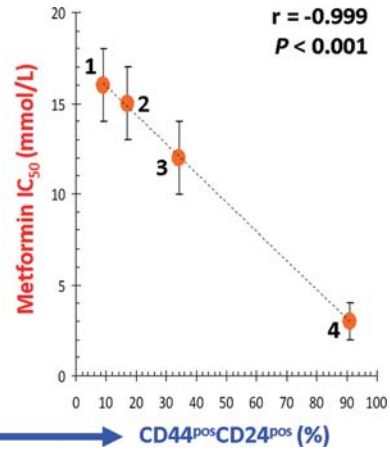

Figure 1. Sensitivity of breast cancer cell cultures to growth inhibitory effects of metformin relates to their relative content in $CD44^{pos}CD24^{pos}$ populations. MCF-7, MDA-MB-231, JIMT-1 and MDA-MB-468 cell cultures were analysed for surface expression of CD24 and CD44 using flow cytometry. The results of this analysis were summarized (%) with respect to four cell population fractions: $CD44^{pos}CD24^{neg}$, $CD44^{neg}CD24^{pos}$, $CD44^{pos}CD24^{pos}$ and $CD44^{neg}CD24^{neg}$. For each cell line, the relative content of each subpopulation was plotted against the metformin's growth inhibitory activity (micromolar IC_{50} value). The figure shows correlation of the $CD44^{pos}CD24^{pos}$ expression vs. IC_{50} values as assessed by linear regression analysis.

Statistical analysis. For correlations between two parameters, the predicted lines were determined by simple linear regression analysis. The P-values and Pearson's linear correlation coefficient (r) were calculated with XLSTAT (Addinsoft™) and P<0.001 was considered to be significant.

Results

Responses of breast cancer cell populations to metformin relate to the relative content of $CD44^{pos}CD24^{pos}$ cells. MTT-based cell viability assays confirmed that, among a wide panel of human breast cancer cell lines (data not shown), MDA-MB-468 cells (the main 'EGFR-positive' breast cancer model of intrinsic resistance to EGFR Tyrosine Kinase Inhibitors that shows many of the recurrent basal-like molecular abnormalities including ER-PR-HER2 triple-negative status, TP53 deficiency, PTEN loss and constitutive activation of the PI-3'K/AKT and MEK/ERK pathways (20,21)) were the most sensitivity ones to the growth-inhibitory effects of metformin. The IC_{50} values (i.e., the concentration of metformin required to decrease MTT metabolization by 50%) ranged from 18±2 mmol/l metformin in low-sensitive luminal MCF-7 BC cells to 3±1 mmol/l metformin in highly-sensitive basal-like MDA-MB-468 breast cancer cells (Fig. 1). Although this 6-fold change in the metformin IC_{50} value might suggest an intrinsic exacerbated response of basal-like breast cancer cells to the growth-inhibitory breast cancer effects of metformin, we also noted that basal-like MDA-MB-231 breast cancer cells were somewhat resistant to metformin in terms of MTT-metabolization.

Since non-invasive MDA-MB-468 cells lack a significant $CD44^{pos}CD24^{neg/low}$ population whereas highly-invasive MDA-MB-231 cells naturally bear >80% of $CD44^{pos}CD24^{neg/low}$ cells (22-24), we decided to investigate the importance of the CD44/CD24 breast cancer immunophenotype in the response to metformin. Interestingly, when IC_{50} values of each cell line for metformin were plotted as a function (on a linear-linear scale) of the relative content of four cell population fractions defined by the expression patterns of CD44 and CD24 (i.e., $CD44^{neg}CD24^{neg}$, $CD44^{neg}CD24^{pos}$, $CD44^{pos}CD24^{pos}$ and $CD44^{pos}CD24^{neg}$), regression analysis showed a strong linear correlation (r=-0.99) between the growth inhibitory effects of metformin and the relative amount of breast cancer cells bearing the $CD44^{pos}CD24^{pos}$ immunophenotype (Fig. 1).

Metformin treatment down-regulates CD24 protein expression in highly-metastatic MDA-MB-468 basal-like breast cancer cells. We next explored whether exacerbated responses of $CD44^{pos}CD24^{pos}$-enriched breast cancer cell cultures related to changes in the expression of CD24 and/or CD44 following exposure to metformin. Indirect immunofluorescence imaging of CD24 and CD44 markers in whole (metformin-refractory) MCF-7 and (metformin-sensitive) MDA-MB-468 breast cancer cell populations growing in individual wells, captured as 3x3 montages using an automated confocal high-content imaging approach, confirmed that metformin efficacy, in terms of breast cancer cell viability, closely correlated with the positivity of CD24 and CD44 stem cell markers in MDA-MB-468 cultures (data not shown). Interestingly, C24 and CD44 protein expression levels were dynamically altered in the presence of metformin. Exogenous supplementation with 10 mmol/l metformin for 48 h yielded a drastic increase in the percentage of $CD24^{neg}$ cells as well a drastic decrease in the median fluorescence intensity relative to untreated MDA-MB-468 cells (Fig. 2A). Moreover, metformin-induced depletion of CD24 was accompanied by a significant up-regulation of CD44 in $CD24^{neg}$ cells (Fig. 2B). In light of these findings it would be reasonable to suggest that metformin treatment accelerates the intrinsic plasticity of non-invasive, epithelial-like $CD44^{pos}CD24^{pos}$ cells to give rise to undesirable invasive, mesenchymal-like $CD44^{pos}CD24^{neg/low}$ progeny. However, metformin-induced depletion of the CD24 oncoprotein in $CD44^{pos}CD24^{pos}$-enriched MDA-MB-468 failed to significantly alter cell morphology and it did not yield a mesenchymal phenotype.

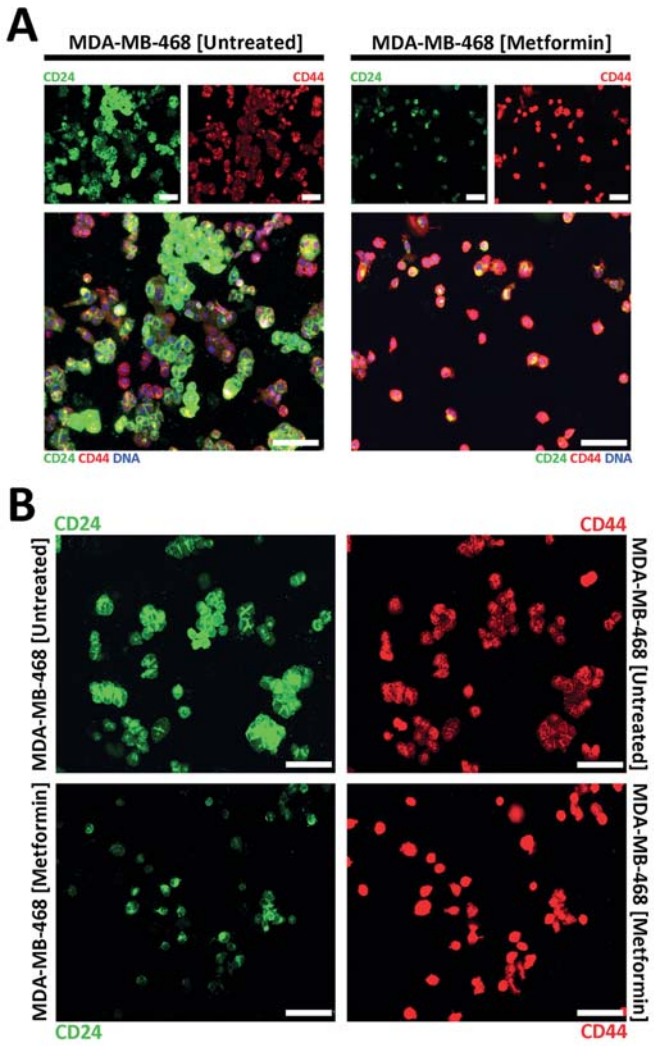

Figure 2. Metformin treatment down-regulates CD24 protein expression in $CD44^{pos}CD24^{pos}$-enriched MDA-MB-468 basal-like breast cancer cells. After fixation and permeabilization, cellular distribution of CD44 and CD24 was assessed following staining with anti-CD44 rabbit antibody (HPA005785; Prestige Antibodies® Powered by Atlas Antibodies, Sigma-Aldrich), anti-CD24 mouse monoclonal antibody (sc-70598; Santa Cruz Biotechnology, Inc.) and Hoechst 33258 for nuclear counterstaining. Images show representative whole population of MDA-MB-468 cells growing in individual wells in the absence or presence of 10 mmol/l metformin for 48 h that were captured using different channels for CD44 (red), CD24 (green) and Hoechst 33258 (blue) as a 3x3 montage with a x20 objective on BD Pathway™ 855 Bioimager System, and merged using BD Attovision™ software. Scale bars, 100 μm.

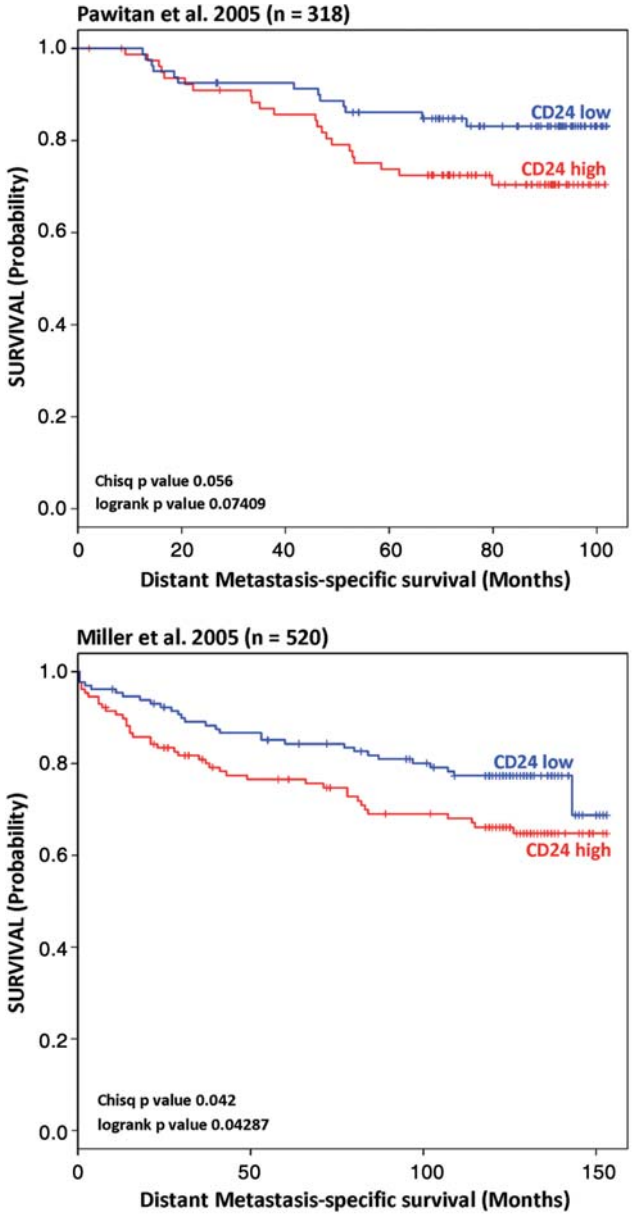

Figure 3. Distant metastasis-free survival (DMSF) according to CD24 expression levels in primary breast carcinomas. Kaplan-Meier survival plots for distant metastasis-specific survival are shown for patients classified according to CD24 expression status. Red branches denote tumors with high levels of CD24 expression; blue branches identify those with the low levels of CD24 expression.

CD24 overexpression relates to shorter distant metastasis-free survival in breast cancer patients. To preliminarily assess the clinical relevance of metformin's anti-CD24 effects we took advantage of the recently developed ROCK online interface (http://rock.icr.ac.uk/), a publicly accessible portal that allows a rapid integration of breast cancer functional and molecular profiling datasets. Rather than to validate the effect of CD24 expression on disease-free survival (DFS) and overall survival (OS) in patients with breast cancer (8), we decided to evaluate an association between CD24 levels and distant metastasis-free survival (DMFS). ROCK employs the Kaplan-Meier method to construct survival curves, and the long-rank test is used to compare survival outcome. Also, χ^2 tests and analysis of variance are used to compare between-group patients' survival according to CD24 expression status. We evaluated the impact of CD24 expression on DMFS using microarray gene expression breast cancer datasets that involved large number of patients (n ≥300). Although results failed to reach statistical significance, gene expression profiles of 318 breast cancer patients provided by Pawitan *et al* (25) suggested a trend toward worse DMFS in CD24-high tumors (Fig. 3, top panel). Kaplan-Meier survival analysis together with log-rank and χ^2 tests comparing DMFS for CD24-high and CD24-low breast carcinomas in 520 breast cancer gene profiles provided by Miller *et al* (26) revealed that those with high CD24 expression had significantly worse DMFS than those with CD24-low tumors (Fig. 3, bottom panel).

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A landmark study by Hirsch and colleagues has recently revealed that tumor-initiating breast cancer (stem) cells, as defined by the $CD44^{pos}CD24^{neg/low}$ immunophenotype, exhibit an exacerbated sensitivity to the growth inhibitory effects of the anti-diabetic biguanide metformin (27). In their hands, low doses of metformin selectively eliminated the $CD44^{pos}CD24^{neg/low}$ cell subpopulation in a series of human breast cancer cell lines. We recently confirmed the ability of metformin to suppress self-renewal and proliferation of HER2-positive CD44-overexpressing tumor-initiating breast cancer stem cells (28). Our current findings strongly suggest that metformin treatment might ablate also the recently documented tumor-initiating potential of $CD44^{pos}CD24^{pos}$ cells (4,5). CD24 has recently generated considerable attention in tumor biology due to its function in cell adhesion (6-8). Accordingly, it has been suggested that CD24 overexpression worsens prognosis because it plays an important role in the metastatic progression of breast cancer disease (29-31). We now reveal that high levels of the CD24 oncoprotein in $CD44^{pos}CD24^{pos}$-enriched highly-metastatic MDA-MB-468 basal-like breast cancer cells are notably decreased following incubation with metformin. Given that non-invasive, epithelial-like $CD44^{pos}CD24^{pos}$ cells can readily give rise to invasive, mesenchymal $CD44^{pos}CD24^{neg}$ progeny, 'anti-CD24 agents' should concomitantly prevent the appearance of epithelial-to-mesenchymal (EMT) phenomena within $CD44^{pos}CD24^{pos}$ populations as they might generate undesirable tumor-initiating $CD44^{pos}CD24^{neg/low}$ stem-like mesenchymal phenotypes. In our hands, the anti-diabetic drug metformin not only functioned as an efficient 'anti-CD24' agent but also appeared to efficiently ablate the tumor-initiating potential of $CD44^{pos}CD24^{pos}$ cells due to its ability to concomitantly repress the EMT genetic program, thus preventing the appearance of mesenchymal-like molecular signatures in basal-like breast cancer cells (unpublished data). Although data presented herein do not rule out metformin-induced regulation of CD24 expression by modified translation or cell surface localization of the protein, our findings are consistent with earlier studies demonstrating that the *CD24* gene is indeed susceptible to dynamic transcriptional regulation. In this regard, it has been suggested that estrogen/ERα functionally regulates putative breast cancer stem cells because CD24 is repressed by estrogen. and this repression is a direct transcriptional effect depending on ERα and histone deacetylases (HDACs), while CD44 is up-regulated by estrogen-bound ERα (11,32,33). It is interesting to note that metformin-induced down-regulation of CD24 was accompanied by a significant up-regulation of CD44, thus suggesting that a CD24/CD44 cross-talk could take place also in ERα-negative breast cancer cells; obviously, it should involve other yet to be explored estrogen/ERα-independent regulatory mechanisms such as epigenetic silencing or involvement of other transcription factors.

Kristiansen *et al* (31) pioneeringly described a significant association of CD24 expression with shortened patient OS and DFS, thus suggesting that CD24 expression in primary breast cancer as detected by immunohistochemistry might a new marker for more aggressive breast cancer biology. A meta-analysis of the relationship between CD24 expression and prognostic parameter in different carcinomas supported the notion that CD24 is an important marker of malignancy and poor prognosis that may promote breast cancer development and progression (8). We here confirm and expand further the notion that CD24 expression might be a useful marker for human breast carcinoma as it could play a role in facilitating metastasis by the interaction between tumor cells and platelets or endothelial cells (30). In CD24-overexpressing breast cancer cells, CD24 appears to be involved in tumor growth and migration because CD24 cross-linking induces (34). It has been repeatedly suggested that primary luminal breast tumors consists of $CD44^{pos}$ stem-like breast cancer cells with *de novo* high invasive and angiogenic capacity, and more differentiated $CD24^{pos}$ cells. In this scenario, distant metastasis can be initiated by invasive $CD44^{pos}$ cells that later can switch to a more differentiated $CD24^{pos}$ cell phenotype due to microenvironmental conditions (hypoxia, stromal factors, etc.) or selection due to therapeutic interventions (3,10). Alternatively, $CD24^{pos}$ cells can drive invasion and metastasis themselves during breast cancer progression and they can change to $CD44^{pos}$ stem cell-like cells upon acquisition of genetic, epigenetic changes or following cell response to environmental factors including hypoxia (3,10). In any case, distant metastases are largely composed of $CD24^{pos}$ more differentiated cells in patients refractory to treatment (9). Of note, MDA-MB-468 cells expressing high levels of both CD44 and CD24 (and with <3% $CD44^{pos}CD24^{neg/low}$ stem cell-like subpopulation) fail to invade but efficiently metastasize to lung and, at sites of metastasis, cancer cell phenotype based on CD44 and CD24 expression does not change significantly compared with that of parental cells (22). Because it is unlikely that few $CD44^{pos}CD24^{neg/low}$ cells of MDA-MB-468 cells (35) could contribute to lung metastasis and then progressed to become $CD44^{pos}CD24^{pos}$, the fact that animals injected with $CD44^{pos}CD24^{pos}$-rich MDA-MB-468 triple-negative cells have severe morbidity and mortality in the absence of bone metastasis (22) strongly suggests that the $CD44^{pos}CD24^{neg/low}$ phenotype, while associated with invasion, is not sufficient to establish pulmonary metastasis. Because our current evaluation of the impact of CD24 expression on DMFS in microarray gene expression breast cancer datasets revealed a significantly shorter DMFS in CD24-high patients, these findings not only warrant additional studies aimed to elucidate the mechanisms of CD24-driven tumor cell growth at sites of metastasis but provide further a molecular rationale to clinically explore the anti-CD24 effects of the anti-diabetic drug metformin during the therapeutic management of highly-metastastic subgroups of triple-negative (basal-like) breast cancers naturally enriched with $CD44^{pos}CD24^{pos}$ tumor-initiating cell populations.

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