

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

ALEJANDRO VAZQUEZ-MARTIN^{1,2*}, CRISTINA OLIVERAS-FERRAROS^{1,2*},
SILVIA CUFÍ^{1,2}, SONIA DEL BARCO^{1,2}, BEGOÑA MARTIN-CASTILLO^{1,2},
EUGENI LOPEZ-BONET^{2,3} and JAVIER A. MENENDEZ^{1,2}

¹Catalan Institute of Oncology (ICO-Girona), ²Girona Biomedical Research Institute (IdIBGi), ³Department of Pathology, Dr Josep Trueta University Hospital of Girona, Avenida de Francia S/N, E-17007 Girona, Catalonia, Spain

Received September 6, 2010; Accepted September 27, 2010

DOI: 10.3892/or_00001052

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-metastatic subgroups of triple-negative (basal-like) breast cancers naturally enriched with CD44^{pos}CD24^{pos} tumor-initiating cell populations.

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

Correspondence to: Dr Javier A. Menendez, Catalan Institute of Oncology (ICO-Girona), Dr Josep Trueta University Hospital, Avenida de Francia S/N, E-17007 Girona, Catalonia, Spain
E-mail: jmenendez@iconcologia.net; jmenendez@idibgi.org

*Contributed equally

Key words: metformin, triple-negative, basal-like, CD24, stem cells, EMT

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 (ER α) 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 were 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% CO₂. 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 μ l 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) \times 100. Breast cancer cell sensitivity to metformin was expressed in terms of the concentration of the drug required to decrease by 50% cell viability (IC₅₀ value). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC₅₀ 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 (10⁶ cells/100 μ l). 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).

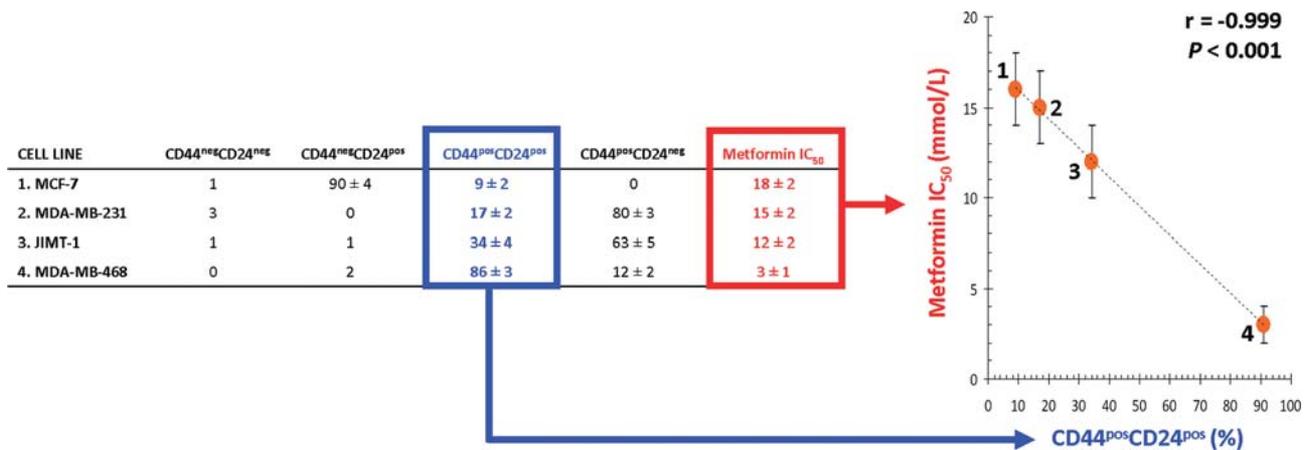


Figure 1. Sensitivity of breast cancer cell cultures to growth inhibitory effects of metformin relates to their relative content in CD44^{pos}CD24^{pos} populations. MCF-7, MDA-MB-231, JIMT-1 and MDA-MB-468 cell cultures were analysed for surface expression of CD24 and CD44 using flow cytometry. The 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 IC₅₀ value). The figure shows correlation of the CD44^{pos}CD24^{pos} expression vs. IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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, CD24 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.

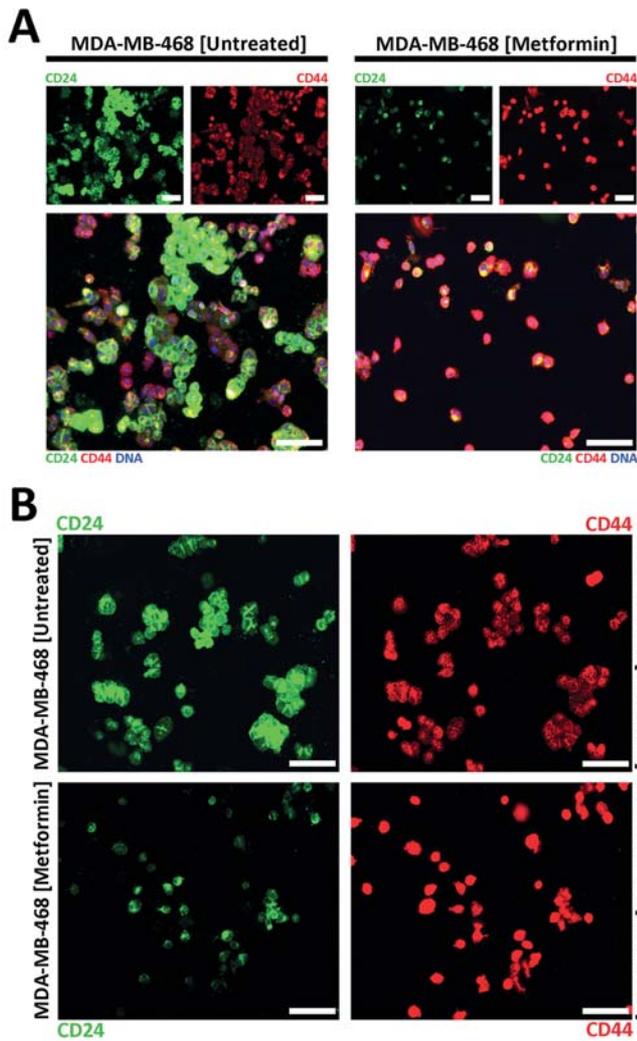


Figure 2. Metformin treatment down-regulates CD24 protein expression in CD44^{pos}CD24^{pos}-enriched MDA-MB-468 basal-like breast cancer cells. After fixation and permeabilization, cellular distribution of CD44 and CD24 was assessed following staining with anti-CD44 rabbit antibody (HPA005785; Prestige 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 μ m.

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,

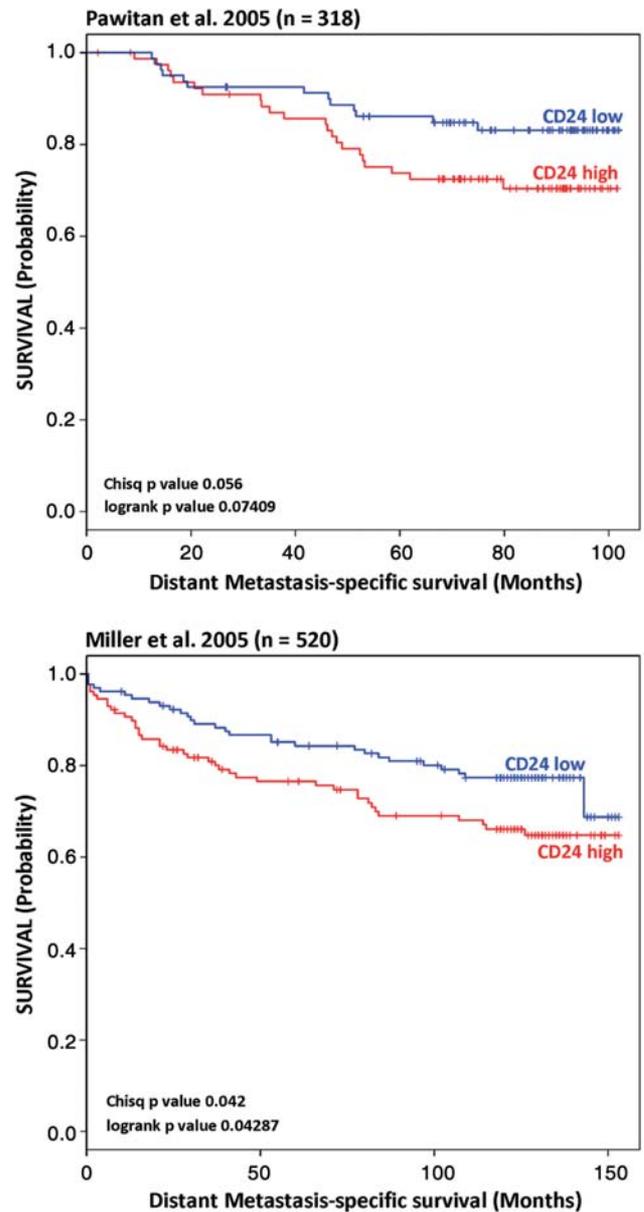


Figure 3. Distant metastasis-free survival (DMFS) according to CD24 expression levels in primary breast carcinomas. Kaplan-Meier survival plots for distant metastasis-specific survival are shown for patients classified according to CD24 expression status. Red branches denote tumors with high levels of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

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 ($n \geq 300$). 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).

Discussion

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/ER α functionally regulates putative breast cancer stem cells because CD24 is repressed by estrogen, and this repression is a direct transcriptional effect depending on ER α and histone deacetylases (HDACs), while CD44 is up-regulated by estrogen-bound ER α (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 ER α -negative breast cancer cells; obviously, it should involve other yet to be explored estrogen/ER α -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 be 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-metastatic subgroups of triple-negative (basal-like) breast cancers naturally enriched with CD44^{pos}CD24^{pos} tumor-initiating cell populations.

Acknowledgements

Alejandro Vazquez-Martin is the recipient of a 'Sara Borrel' post-doctoral contract (CD08/00283, Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria -FIS-, Spain). Research work at Menendez' Laboratory is supported in part by the Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria -FIS-, Spain,

Grants CP05-00090, PI06-0778 and RD06-0020-0028), the Fundación Científica de la Asociación Española Contra el Cáncer (AECC, Spain), and by the Ministerio de Ciencia e Innovación (SAF2009-11579, Plan Nacional de I+D+I, MICINN, Spain).

References

- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 100: 3983-3988, 2003.
- Nakshatri H, Srouf EF and Badve S: Breast cancer stem cells and intrinsic subtypes: controversies rage on. *Curr Stem Cell Res Ther* 4: 50-60, 2009.
- Shipitsin M and Polyak K: The cancer stem cell hypothesis: in search of definitions, markers, and relevance. *Lab Invest* 88: 459-463, 2008.
- Meyer MJ, Fleming JM, Ali MA, Pesesky MW, Ginsburg E and Vonderhaar BK: Dynamic regulation of CD24 and the invasive, CD44^{pos}CD24^{neg} phenotype in breast cancer cell lines. *Breast Cancer Res* 11: R82, 2009.
- Lewis MT: The more things change ... the more things change: developmental plasticity of tumor-initiating mammary epithelial cells. *Breast Cancer Res* 12: 101, 2010.
- Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, Uede T, Yagita H and Sleeman JP: CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. *Cancer Res* 65: 10783-10793, 2005.
- Lim SC: CD24 and human carcinoma: tumor biological aspects. *Biomed Pharmacother* 59 (Suppl. 2): S351-S354, 2005.
- Lee JH, Kim SH, Lee ES and Kim YS: CD24 overexpression in cancer development and progression: a meta-analysis. *Oncol Rep* 22: 1149-1156, 2009.
- Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS and Polyak K: Molecular definition of breast tumor heterogeneity. *Cancer Cell* 11: 259-273, 2007.
- Polyak K, Shipitsin M, Campbell-Marrotta L, Bloushtain-Qimron N and Park SY: Breast tumor heterogeneity: causes and consequences. *Breast Cancer Res* 11 (Suppl. 1): S18, 2009.
- Kaipappattu BA, Malik S, Konduri SD, Liu W, Rokavec M, van der Kuip H, Hoppe R, Hammerich-Hille S, Fritz S, Parker W, Abele I, Das GM, Oesterreich S and Brauch H: Estrogen-mediated down-regulation of CD24 in breast cancer cells. *Int J Cancer* 123: 66-72, 2008.
- Surowiak P, Materna V, Paluchowski P, Matkowski R, Wojnar A, Maciejczyk A, Pudelko M, Kornafel J, Dietel M, Kristiansen G, Lage H and Zabel M: CD24 expression is specific for tamoxifen-resistant ductal breast cancer cases. *Anticancer Res* 26: 629-634, 2006.
- Kim HJ, Kim JB, Lee KM, Shin I, Han W, Ko E, Bae JY and Noh DY: Isolation of CD24^{high} and CD24^{low/-} cells from MCF-7: CD24 expression is positively related with proliferation, adhesion and invasion in MCF-7. *Cancer Lett* 258: 98-108, 2007.
- Martin-Castillo B, Vazquez-Martin A, Oliveras-Ferraros C and Menendez JA: Metformin and cancer: doses, mechanisms and the dandelion and hormetic phenomena. *Cell Cycle* 9: 1057-1064, 2010.
- Vazquez-Martin A, Oliveras-Ferraros C, Cufí S, Martin-Castillo B and Menendez JA: Metformin and energy metabolism in breast cancer: from insulin physiology to tumour-initiating stem cells. *Curr Mol Med* 10: 1-18, 2010.
- Liu B, Fan Z, Edgerton SM, Deng XS, Alimova IN, Lind SE and Thor AD: Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle* 8: 2031-2040, 2009.
- Jiralerspong S, Gonzalez-Angulo AM and Hung MC: Expanding the arsenal: metformin for the treatment of triple-negative breast cancer? *Cell Cycle* 8: 2681, 2009.
- Rakha EA, Reis-Filho JS and Ellis IO: Basal-like breast cancer: a critical review. *J Clin Oncol* 26: 2568-2581, 2008.
- Sims D, Bursteinas B, Gao Q, Jain E, Mackay A, Mitsopoulos C and Zvebil M: ROCK: a breast cancer functional genomics resource. *Breast Cancer Res Treat*: June 20, 2010 (Epub ahead of print).
- Yehiely F, Moyano JV, Evans JR, Nielsen TO and Cryns VL: Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med* 12: 537-544, 2006.
- Oliveras-Ferraros C, Vazquez-Martin A, López-Bonet E, Martín-Castillo B, Del Barco S, Brunet J and Menendez JA: Growth and molecular interactions of the anti-EGFR antibody cetuximab and the DNA cross-linking agent cisplatin in gefitinib-resistant MDA-MB-468 cells: new prospects in the treatment of triple-negative/basal-like breast cancer. *Int J Oncol* 33: 1165-1176, 2008.
- Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R Jr, Badve S and Nakshatri H: CD44⁺/CD24⁻ breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* 8: R59, 2006.
- Honeth G, Bendahl PO, Ringnér M, Saal LH, Gruvberger-Saal SK, Lövgren K, Grabau D, Fernö M, Borg A and Hegardt C: The CD44⁺/CD24⁻ phenotype is enriched in basal-like breast tumors. *Breast Cancer Res* 10: R53, 2008.
- Oliveras-Ferraros C, Vazquez-Martin A, Martin-Castillo B, Cufí S, Del Barco S, Lopez-Bonet E, Brunet J and Menendez JA: 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). *Biochem Biophys Res Commun* 397: 27-33, 2010.
- Pawitan Y, Bjöhle J, Amler L, Borg AL, Egyhazi S, Hall P, Han X, Holmberg L, Huang F, Klaar S, Liu ET, Miller L, Nordgren H, Ploner A, Sandelin K, Shaw PM, Smeds J, Skoog L, Wedrén S and Bergh J: Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. *Breast Cancer Res* 7: R953-R964, 2005.
- Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET and Bergh J: An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci USA* 102: 13550-13555, 2005.
- Hirsch HA, Iliopoulos D, Tschlis PN and Struhl K: Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* 69: 7507-7511, 2009.
- Vazquez-Martin A, Oliveras-Ferraros C, Barco SD, Martin-Castillo B and Menendez JA: The anti-diabetic drug metformin suppresses self-renewal and proliferation of trastuzumab-resistant tumor-initiating breast cancer stem cells. *Breast Cancer Res Treat*: May 11, 2010 (Epub ahead of print).
- Lim SC and Oh SH: The role of CD24 in various human epithelial neoplasias. *Pathol Res Pract* 201: 479-486, 2005.
- Fogel M, Friederichs J, Zeller Y, Husar M, Smirnov A, Roitman L, Altevogt P and Shoeger ZM: CD24 is a marker for human breast carcinoma. *Cancer Lett* 143: 87-94, 1999.
- Kristiansen G, Winzer KJ, Mayordomo E, Bellach J, Schlüns K, Denkert C, Dahl E, Pilarsky C, Altevogt P, Guski H and Dietel M: CD24 expression is a new prognostic marker in breast cancer. *Clin Cancer Res* 9: 4906-4913, 2003.
- Sorbello V, Fuso L, Sfiligoi C, Scafoglio C, Ponzzone R, Biglia N, Weisz A, Sismondi P and De Bortoli M: Quantitative real-time RT-PCR analysis of eight novel estrogen-regulated genes in breast cancer. *Int J Biol Markers* 18: 123-129, 2003.
- Durst B, Sorg RV, Röder G, Betz B, Beckmann MW, Niederacher D, Bender HG and Dall P: The influence of hormones on CD44 expression in endometrial and breast carcinomas. *Oncol Rep* 8: 987-993, 2001.
- Kim JB, Ko E, Han W, Lee JE, Lee KM, Shin I, Kim S, Lee JW, Cho J, Bae JY, Jee HG and Noh DY: CD24 cross-linking induces apoptosis in, and inhibits migration of, MCF-7 breast cancer cells. *BMC Cancer* 8: 118, 2008.
- Agelopoulos K, Greve B, Schmidt H, Pospisil H, Kurtz S, Bartkowiak K, Andreas A, Wiczorek M, Korsching E, Buerger H and Brandt B: Selective regain of egfr gene copies in CD44⁺/CD24^{low} breast cancer cellular model MDA-MB-468. *BMC Cancer* 10: 78, 2010.