

Membrane proteome functional characterization of breast cancer-initiating cells subjected to bone morphogenetic protein signaling inhibition by dorsomorphin

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Abstract In this study, A17 cells, which are an invasive mesenchymal cell line with cancer stem cell properties, were exploited for the study of the role of bone morphogenetic protein pathways in cancer-initiating cells employing a proteomics-based approach. A17 cells were treated with the bone morphogenetic protein signaling inhibitor dorsomorphin for 3 days. After that, subcellular fractionation of cell samples was performed and the membrane fraction analyzed by shotgun proteomics. The extracted membrane proteins were enzymatically digested and the resulting peptide mixture was analyzed by nano liquid chromatography coupled to tandem mass spectrometry and relative label-free quantitation. Protein profiles of A17 membrane fractions before and after dorsomorphin treatment were compared, and further mined by Gene Ontology search. The protein profile of untreated A17 samples correlated with the mesenchymal phenotype, whereas changes were observed in dorsomorphin-treated samples, further supporting a mesenchymal to epithelial transition upon bone morphogenetic protein signaling pathway inhibition and the importance of this pathway in breast cancer cell malignancy.

Keywords Breast cancer · Mesenchymal-epithelial transition · Dorsomorphin · Proteomics · Spectral counting ·

Mass spectrometry · Bone morphogenetic protein signaling inhibition

Introduction

Stem cells have a critical function in tumorigenesis due to their capacity of unlimited self-renewal, proliferation, and long life, which may allow them to accumulate mutations for tumorigenic transformation and cancer initiation (White and Lowry, 2015). According to the cancer stem cell hypothesis for tumor development and progression, cancer stem cells are just a minority of tumor cell population, but they are able to recapitulate the original tumor and maintain tumor growth indefinitely (Regenbrecht et al., 2008).

Cancer growth also depends on the presence of an active stroma that gives vascular and structural support; for breast cancer, myofibroblasts are thought to play a critical role, both for tumor invasion and physiological wound repair. A17 cells are a myofibroblast-like reactive stromal cell line isolated from mammary tumors of epithelial origin, spontaneously developed in FVB/neuT transgenic mice (Galiè et al., 2005). A17 cells generate mesenchymal tumors and show all features of cancer-initiating cells, such as high tumorigenicity and invasion, mesenchymal characteristics, and stem traits (i.e., expression of stem markers and mammosphere formation). In a comparative gene expression analysis, A17 cells were compared to normal mesenchymal stem cells, pointing out that the transcriptome for 258 genes differed for only very few genes, which had a minimum two-fold difference in expression. Among them the bone morphogenetic protein 4 (BMP4) gene was significantly overexpressed in A17 cells. This result was

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connected to a possible function of this gene in the observed tumorigenic activity of these cells (Galiè et al., 2008). In recent years it has been found that the BMP family is involved in embryogenesis processes, tissue homeostasis, and regulation of cellular expansion, differentiation, apoptosis, and migration in stem and progenitor cells. BMPs bind to two types of transmembrane Ser/Thr kinase receptors, type I and type II receptors, which regulate pathways whose malfunction causes several diseases (bone disease, vascular diseases, organ dystrophies, cancer, and metastasis) (Boergermann et al., 2010; Thawani et al., 2010).

A17 cells express all the molecular components of transmembrane Ser/Thr kinase receptors type I and type II required for BMP signaling. The activation of the intracellular signaling pathways downstream the receptors has also already been assessed by western blotting. Therefore, these cells appeared to be a suitable model to study the role of BMP-activated pathways in cancer-initiating cells (Garulli et al., 2014).

At present BMP signaling has been reported to be involved for several cancers; however, its function is still unclear and results contradicting (Thawani et al., 2010; Ketolainen et al., 2010). Moreover, BMP signaling has also been demonstrated to have a critical function in various stem cell populations (Dutko and Mullins, 2011). For these reasons it may be involved also in cancer stem cells, and additional information may help to elucidate what determines the difference between pluripotency and tumorigenicity.

In particular, in this work the BMP signaling modulation was investigated using dorsomorphin (DM), which is a specific inhibitor of this pathway (Hong and Yu, 2009). Previous studies on the A17 cell line have demonstrated DM capability to reduce signal transduction in the BMP pathway in a dose-dependent manner (Garulli et al., 2014).

For the present work, DM effect on BMP signaling pathway was investigated by a shotgun proteomics approach, chosen as a screening methodology because it is sensitive and accurate enough to allow the study of global changes in protein abundances also in complex biological systems, giving the possibility to investigate the response to perturbations (Hu et al., 2005; Han et al., 2008; Gilmore and Washburn, 2010). In particular, A17 cells were treated with DM and the membrane fraction was studied to characterize the proteome and individuate changes at protein level. The membrane fraction was chosen due to the many regulatory functions possessed by membrane proteins, including: signal transduction, intercellular communication, biogenesis, cell motility, and molecular transport. Membrane protein fractions were obtained by a solubility-based protein fractionation protocol. Then, samples were processed according to shotgun proteomics with in-solution tryptic digestion and nano liquid chromatography-tandem mass spectrometry

(nanoLC-MS/MS) analysis. Moreover to provide a more comprehensive description of the changes occurring during the analysis, a quantitative label-free analysis was also performed (Old et al., 2005; Neilson et al., 2011). Additionally, Gene Ontology (GO) was used to retrieve information about the identified proteins. The qualitative protein profiles and quantitative protein abundance differences were then discussed and related to BMP pathway and compared to previous results for the same system obtained with investigation approaches other than proteomics (Garulli et al., 2014).

Material and methods

Chemicals and reagents

DMEM (Dulbecco's Modified Eagle Medium) (Invitrogen), fetal bovine serum (FBS) (Gibco), and 100 U/mL penicillin-100 mg/mL streptomycin (Gibco) were purchased from Invitrogen (Paisley, UK). DM, dimethylsulfoxide (DMSO), ammonium bicarbonate, trifluoroacetic acid (TFA), formic acid, and Bradford protein assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitor mix, tris (hydroxymethyl) aminomethane, dithiothreitol (DTT), iodoacetamide (IAA), and urea were purchased from GE Healthcare (Uppsala, Sweden). Qproteome Cell Compartment Kit was purchased from Quiagen (Valencia, CA, USA). Modified porcine trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA). Solid phase extraction (SPE) C18 cartridges (Bond Elut 1cc LRC-C18) were purchased from Varian (Palo Alto, CA, USA). ReproSil-Pur C18-AQ 3 μm resin was purchased from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). All organic solvents were the highest grade available from Carlo Erba Reagents (Milan, Italy) and were used without any further purification. Ultrapure water was produced from distilled water by a Milli-Q system (Millipore Corporation, Billerica, MA, USA).

Cell culture and stimulation

A17 cells were obtained from spontaneous tumors isolated from HER-2/neu transgenic mice as previously described (Galiè et al., 2005). Cell cultures were maintained in DMEM supplemented with 20 % FBS, 100 U mL⁻¹ penicillin-100 mg mL⁻¹ streptomycin. Cells to be treated with DM were trypsinized from a confluent flask and two billion of them were treated with 2 $\mu\text{mol L}^{-1}$ DM in DMSO supplemented on normal medium. Cells were kept in suspension and plated on 25 cm² flask in the media containing the inhibitor and grown up for 3 days.

Cells treated with media containing an equivalent amount of DMSO were used as a negative control. Media containing either DM or DMSO were changed daily for 3 days. DMSO-treated cells had similar outcomes to control untreated cells, indicating that DMSO had no effect on A17 cell growth.

Subcellular fractionation and protein extraction

All steps of the extraction procedure were performed on ice using pre-chilled solutions. Centrifugation and incubation were carried out at 4 °C. All buffers were supplemented with protease inhibitor before use. Protein extraction from cells was performed by using the Qproteome Cell Compartment Kit according to the manufacturer protocol for subcellular extraction of proteins. After membrane fraction isolation, the protein content was determined using a Bradford protein assay against BSA diluted in solubilization buffer. Three biological replicates were performed and the protein estimations were carried out in triplicates.

Protein digestion

Membrane proteins (50 µg) of both samples (A17 and DM-treated A17 cells) were precipitated with chloroform/methanol protein precipitation as already described (Wessel and Flugge, 1984). Pellets were air-dried and then digested as described below. First, proteins were denatured redissolving the pellets in 40 µL of 8 mol L⁻¹ urea in 50 mmol L⁻¹ ammonium bicarbonate. Then disulphide bonds were reduced adding 2 µL of 200 mmol L⁻¹ DTT solution in 50 mmol L⁻¹ ammonium bicarbonate, under slight agitation, and incubated at 37 °C for 1 h. Carbamidomethylation of thiol groups was performed by addition of 8 µL of 200 mmol L⁻¹ IAA solution in 50 mmol L⁻¹ ammonium bicarbonate, and incubated for 30 min in the dark at room temperature. To consume any leftover alkylating reagent and avoid trypsin alkylation, additional 8 µL of DTT solution was added and samples were incubated at 37 °C for 1 h, under slight agitation. The samples were then diluted with 50 mmol L⁻¹ ammonium solution to obtain a final concentration of 1 mol L⁻¹ urea; sequencing grade-modified trypsin was added (1:20 enzyme to protein ratio) and the samples were incubated overnight at 37 °C. Enzymatic digestion was quenched with formic acid.

Digested samples were desalted using SPE C18 cartridges conditioned with acetonitrile and rinsed with 0.1 % TFA. Peptides were eluted from the SPE column with 500 µL acetonitrile:water (50:50, v/v) containing 0.05 % TFA and were dried in a Speed-Vac SC 250 Express (Thermo Savant, Holbrook, NY, USA). Each sample was re-constituted with 500 µL of 0.1 % formic acid solution and stored at -80 °C until analysis.

NanoLC-MS/MS analysis

Tryptic peptides were analyzed by a Dionex Ultimate 3000 nano-LC system (Sunnyvale, CA, USA) connected with a linear quadrupole ion trap-orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanospray ion source. Instrument calibration was performed employing commercial calibration solutions (Pierce LTQ ESI Positive Ion Calibration Solution, Thermo Scientific, Bremen, Germany) once a week for the Orbitrap and once a month for LTQ.

Peptide mixtures were online enriched injecting 10 µL aliquot of sample on a 300 µm i.d. ×5 mm Acclaim PepMap 100 C18 (5 µm particle size, 100 Å pore size) µ-precolumn (Dionex), employing a premixed mobile phase of ddH₂O: acetonitrile, 98:2 containing 0.1 % (v/v) formic acid (phase C), at a flow rate of 10 µL min⁻¹.

Peptides were separated on an in-house manufactured 20-cm fritless silica microcolumn (75 µm i.d.), packed with ReproSil-Pur C18-AQ 3-µm resin. The column, the whole HPLC system, and MS/MS equipment were tested prior to use for performance, analyzing a commercial standard tryptic digest of *E. Coli* (MassPREP *E. Coli* Digest Standard, Waters, Milford, MA, USA) and considering the chromatogram intensity, chromatogram quality, and the number of identified peptides and proteins. Samples were analyzed with a flow rate of 250 nL min⁻¹ and the LC gradient was optimized to detect the largest set of peptides, using ddH₂O with 0.1 % formic acid (v/v) as phase A and acetonitrile with 0.1 % formic acid (v/v) as phase B. After an isocratic step at 5 % B for 5 min, B was linearly increased to 30 % within 75 min; afterwards, B was increased to 80 % within 5 min, and to 95 % within the following 10 min to rinse the column. Finally, B was lowered to 5 % over 1 min and the column re-equilibrated for 24 min (120 min total run time). MS spectra were collected over an *m/z* range of 400–1800 Da setting Orbitrap resolution at 60,000 (FWHM at *m/z* 400) resolution, operating in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS low resolution acquisition. MS/MS spectra were collected for the five most abundant ions in each MS scan using a dynamic exclusion limit of two MS/MS spectra of a given mass for 30 s with an exclusion duration of 100 s. Collision-induced dissociation was performed with normalized collision energy set at 35 V. In order to assess the additional variation introduced into the measurements by the experimental procedure and to increase the number of identified proteins, we performed five technical replicates (LC-MS/MS runs) for each of the three biological replicates.

Proteins identification

Prior to spectra processing, proper tryptic digestion was assessed by RawMeat software (VAST Scientific, www.rawmeat.com).

vastscientific.com) checking the charge distribution of identified molecules, and a maximum for +2 charge. Then, raw MS/MS data files from Xcalibur software (version 2.0.7 SP1, Thermo Fisher) were submitted to Mascot Daemon (version 2.3.2, Matrix Science) for database search against SwissProt release 2011_10 database (19 October 2011). ThermoFinnigan LCQ/DECA RAW file data import filter was used. The search was limited to *Mus musculus* (house mouse) taxonomy entries and performed using the built-in decoy search option of Mascot. Enzymatic digestion with trypsin was selected, with maximum two missed cleavages, peptide charges +2 and +3, a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.8 Da; acetylation (N-term), oxidation (M), and deamidation (N, Q) were used as dynamic modifications; carbamidomethylation (C) was used as static modification.

Scaffold analysis

Scaffold software (Searle, 2010) (version 3.1.2, Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications, and for label-free relative quantitation based on spectral counting. The additional X! Tandem (Craig and Beavis, 2004) search engine (The GPM, Cyclone 2010.12.01.1 version) was also chosen, keeping the same parameters previously used for Mascot. According to the Peptide and Protein Prophet algorithms (Keller et al., 2002; Nesvizhskii et al., 2003), the peptide probability was set to minimum 95 %, whereas the protein probability was set at 99 %, with at least one identified unique peptide, resulting in a false discovery rate (FDR) for peptides and proteins < 0.1 % (no decoy hits were found). Proteins that contained shared peptides and could not be differentiated based on MS/MS analysis alone were grouped together. Fisher's exact test was used to identify statistically significant differences between A17 and DM-treated samples. Proteins that had at least a two-fold difference for the mean ratio, as well as a Fisher's *p*-value ≤ 0.05 and a relative standard deviation of biological replicates < 40 % were considered present in the two samples in significant different quantities.

Functional classification

GO data about the biological process of identified proteins were obtained by means of Scaffold's built-in option.

Results and discussion

Analytical method

Proper sample preparation for MS-based analysis is a critical step in proteomics workflows because it finalizes analytical results, which in turn determine the biological

significance of the experiment. One issue that complicates protein analysis is the complexity of the mixture, in which many proteins are present in a broad dynamic range of concentration (Feist and Hummon, 2015). A possible strategy to overcome this limitation is the fractionation of the cell into subcellular organelles and compartments before proteomic analysis, which allows to increase the number of identified proteins, also at low concentration. Moreover, additional information also derives from the knowledge of protein localization. An effective separation of tryptic peptides is also needed. In gradient chromatography this can be achieved by the use of a longer columns, also in combination with increasing gradient time, which allows an improvement of peptide separation and protein identification (Liu et al., 2007). For the reasons stated above, in order to obtain high specificity and reproducibility while being cost and time efficient, we have adapted a commercially available kit from Qiagen for proteins separation. Therefore, in this study we decided to combine the subcellular fractionation of A17 and DM-treated cell samples with the implement of a 20-cm long chromatographic column for enhanced peptides separation. To assess the accuracy, reproducibility, and reliability of protein identifications, three biological replicates and five technical replicates were performed. In order to check the correspondence among technical replicates, the unweighted spectral counts of each replicate from the same biological sample were plotted against each other and evaluated using Scaffold's built-in option (an example of scatter plot is reported in Fig. 1, all other plots are available in the Supplementary information, Fig. S1). High squared Pearson correlation values were obtained for both technical ($R^2 = 0.912\text{--}0.966$) and biological replicates ($R^2 = 0.952\text{--}0.990$).

As described, the analytical method provided a total of 1052 protein groups, identified and validated from 132,177 spectra with a FDR for both proteins and peptides < 0.1 %. In particular, 879 protein groups validated for A17 samples and 931 for DM-treated cells (Fig. 1). Most of them (72 %) are common to A17 nontreated and DM-treated samples. On the other hand, 121 proteins (11 %) were exclusively found in A17 samples (A17 unique proteins), whereas 173 (16 %) were exclusively found in DM-treated cells (DM-treated cells unique proteins, Fig. 2). However, quantitative differences were observed for 4 % of the common proteins, with 24 of them being overexpressed in DM-treated cells and 23 underexpressed in DM samples. The list of validated proteins and the related statistical details is listed in Supplementary information, Table S1.

A17 cells membrane protein profile and correlation with the mesenchymal phenotype

The shotgun proteomics results have been used to understand if A17 membrane protein profile showed any

Fig. 1 **a** Example of correlation graph for A17 biological replicate 1, technical replicate 1 vs. 2 (A17_1_1 vs. A17_1_2); **b** Example of correlation graph for A17 biological replicates 1 vs. 2 (A17_1 vs. A17_2)

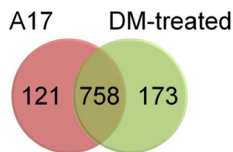
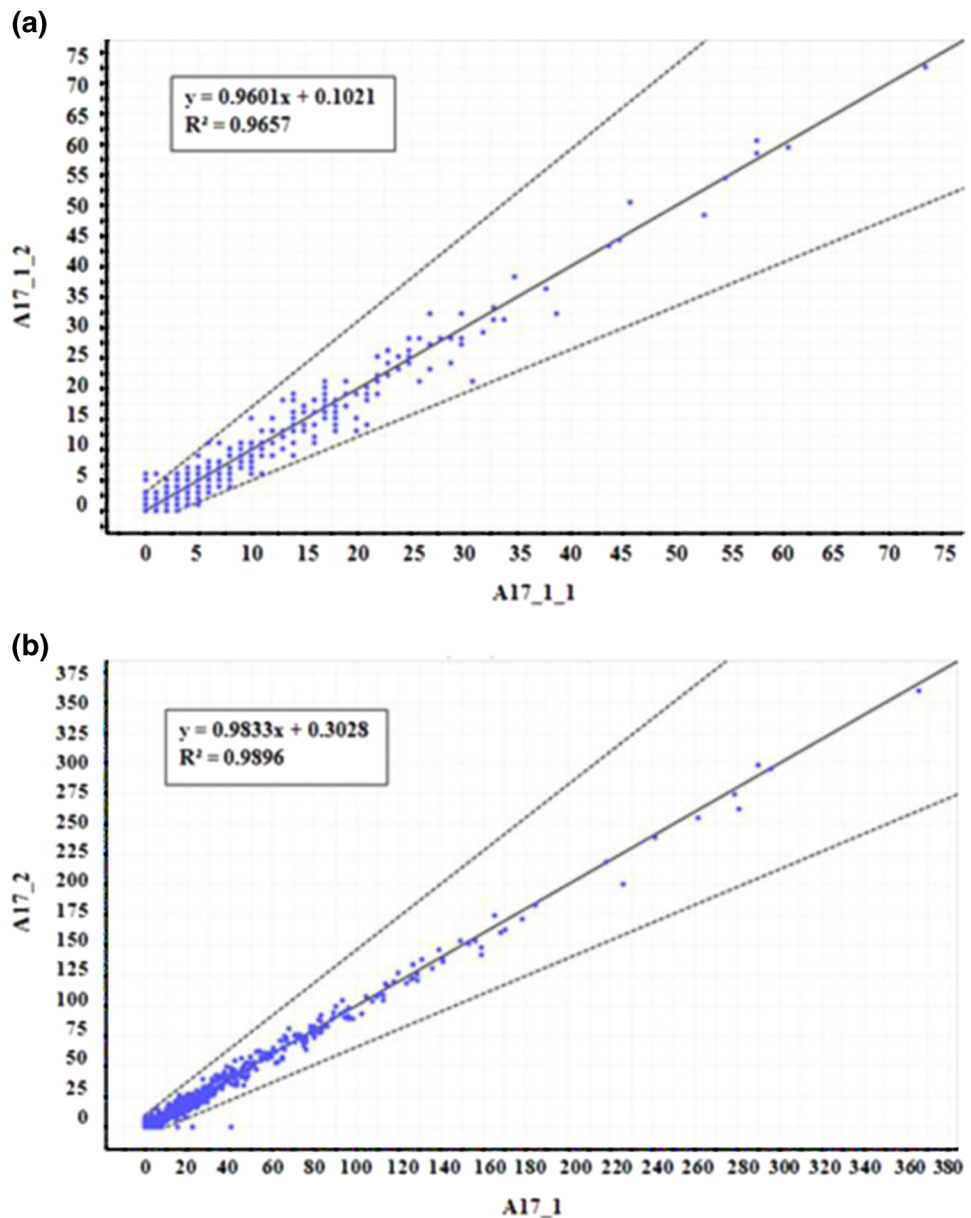


Fig. 2 Venn diagram showing the validated proteins for A17 cell line and DM-treated cells

significant correlation with available genomic data about A17 characterization and with the mesenchymal phenotype; on the other hand, the identified proteins were also used to understand what the effect of DM treatment was upon these cells, in particular considering a possible mesenchymal to

epithelial transition as suggested by previous works (Garulli et al., 2014).

A17 cells share several features with mesenchymal and stem cells and are able to produce mesenchymal tumors. From this point we initially evaluated if A17 cells correlated with the known phenotype that has been described in the literature. From previous studies (Galiè et al., 2005) and characterization of A17 mesenchymal tumors, it was observed that this cell line showed a great proangiogenic potential, supporting tumor growth with the development of new blood vessels. The analysis of the angiogenesis-related genes expressed in A17 cells revealed the presence of important genes involved, which are expected to have influences on the proteome level. Indeed, we found out that

Table 1 Biologically relevant proteins for A17 characterization

Protein name	Entry ID	Ratio DM-treated/A17 cells
Mesenchymal markers		
Actin, alpha cardiac muscle 1	ACTC	1.15
Cadherin-2	CADH2	0.81
Fibronectin	FINC	0.85
Vimentin	VIME	1.82
Mesenchymal-correlated proteins		
Basigin	BASI	A17 unique
Angiogenesis-related gene products found in A17		
Epidermal growth factor receptor	EGFR	1.50
Glutathione peroxidase 1	GPX1	A17 unique
Beta-type platelet-derived growth factor receptor	PGFRB	0.34
Prostaglandin G/H synthase 1	PGH1	0.85
Prostaglandin G/H synthase 2	PGH2	1.63
Inactive tyrosine-protein kinase 7	PTK7	A17 unique
SPARC	SPRC	1.31
Thrombospondin-1	TSP1	A17 unique
Stem cell-related gene products found in A17		
Cathepsin L1	CATL1	A17 unique
Catenin alpha-1	CTNA1	1.32
Catenin beta-1	CTNB1	1.27
Cystatin-C	CYTC	A17 unique
Solute carrier family 2, facilitated glucose transporter member 1	GTR1	A17 unique
Integrin alpha-5	ITA5	0.27
Integrin alpha-V	ITAV	0.69
Integrin beta-1	ITB1	1.36
Integrin beta-5	ITB5	A17 unique
Protein LAP2	LAP2	A17 unique
Neural cell adhesion molecule 1	NCAM1	0.32
Nestin	NEST	16.00

Proteins characterizing A17 cells identified in the experiments are reported and divided as per functions. For each protein, the last column reports the ratio of DM-treated to A17 cells (measured as spectral counting) or if the protein is A17 unique

related proteins were actually present in our control A17 cells (Table 1). In particular, the angiogenesis-related gene products identified were platelet-derived growth factor receptor beta, which was overexpressed in A17 samples, prostaglandin G/H synthase 1, prostaglandin G/H synthase 2, epidermal growth factor receptor, SPARC, and thrombospondin-1 (TSP1). Other two A17 unique proteins were related to angiogenesis based on the GO data, i.e., glutathione peroxidase 1, a protein involved in cell growth and development that promotes blood vessel endothelial

cell migration, and inactive tyrosine-protein kinase 7 (PTK7), which is also involved in other important roles, such as in cell adhesion, cell migration, cell polarity, proliferation, actin cytoskeleton reorganization, and apoptosis (Table 1).

Integrins represent a borderline class of proteins because significantly related to the angiogenic potential but to another important feature as well, stem cell similarities (Table 1). One of them has been identified among the unique proteins of A17 samples, integrin beta-5 (ITB5), but others significant integrins were also found in common with DM-treated cells. Integrins link the extracellular matrix to the cytoskeleton and are involved in regulation of tumor cell invasion and tumor stem cell development. The heterodimer integrin alpha-5/integrin beta-1 is associated with the deposition of and adhesion to fibronectin, a mesenchymal marker characteristic of invasive breast carcinoma cells (Hill et al., 2009). The association of ITB5 and integrin alpha-V (ITAV, also identified in this experiment) is a membrane receptor for fibronectin, metalloproteinase-2, and TSP1. ITAV was particularly important for another aspect, because connected with the typical mesenchymal feature of focal adhesion, and accordingly it has been found to be overexpressed in A17 cells with respect to DM-treated cells.

In the same manner to angiogenesis-related proteins, to further establish a correlation between the data already available for A17 cells and our proteomic data, we continued to search for mesenchymal stem cell gene products present in our lists. In particular, among the unique proteins, there were four important ones: cathepsinL1, cystatin-C, facilitated glucose transporter member 1, and protein LAP2. Other stem cell-related gene products were identified among the common proteins, namely, catenin alpha-1 (CTNA1), catenin beta-1 (CTNB1), neural cell adhesion molecule 1, and nestin.

Apart from relating our proteomic data to genomic data available for A17 cells, we also tried to check for the presence of proteins connected with the mesenchymal phenotype. Considering the whole list of proteins found for A17 samples, and especially those differently expressed between A17 and DM-treated cells, i.e., the 121 proteins exclusively found in A17 cells and those that were overexpressed in A17 cells, we found out that several markers and mesenchymal-correlated proteins could be found, whereas other proteins could also be considered biologically important because connected with the mesenchymal features, such as reduced cell polarity, high migratory capability and invasiveness, enhanced resistance to apoptosis (Table 1) (Hill et al., 2009; Mathias and Simpson, 2009; Kalluri and Weinberg, 2009). Basigin is such a protein: identified among the unique proteins, it is an integral membrane receptor belonging to the immunoglobulin superfamily; it plays several different and important roles in

immunologic phenomena, differentiation, development, and tumor invasiveness by stimulating production of matrix metalloproteinases by fibroblasts (Kanekura et al., 2002). Taking into consideration also the common proteins, the mesenchymal markers found were four, in particular a very important one was cadherin-2 N-cadherin, a calcium-dependent cell adhesion protein belonging to the cadherin family, a group of proteins that usually have hemophilic interactions participating in cell–cell connections. A similar result has been found for fibronectin, a protein that binds cell surfaces and various compounds (such as collagen, fibrin, heparin, DNA, and actin) and plays an important role in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape. It is considered a mesenchymal cell marker; however, it is found in other types of cells as well, such as fibroblasts, epithelial, and other cell types. The identification of fibronectin in A17 was also supported by another unique protein, gelsolin (see Supplementary information), a protein that binds to fibronectin. Actin alpha cardiac muscle and vimentin were other mesenchymal markers identified in the experiment.

Other unique proteins could be connected with mesenchymal features (Supplementary information). One of them was collagen alpha-1(XII) chain that has a cell–cell adhesion function. A second one was growth arrest-specific protein, involved in developmental growth as a positive regulator of epithelial cell proliferation and a specific growth arrest protein for growth suppression and programmed cell death. Another interesting unique protein is myosin-9, involved in cell–cell adherence, cytokinesis, cell shape, and specialized functions such as secretion and capping, but also angiogenesis and blood vessel endothelial cell migration.

DM-treated cells membrane protein profile and induced changes

Established the correlation present between our proteomic results and data available for A17 cells, we tried to understand if the effect of DM treatment upon this cell line was consistent with an expected mesenchymal to epithelial transition, as suggested by recent findings. In fact it was already demonstrated that the BMP pathway is fundamental for the maintenance of the mesenchymal stem cell phenotype (Garulli et al., 2014). Therefore, deep changes were expected at the protein level as well. However, an aspect that could immediately be highlighted was that few expected epithelial markers were lacking, such as E-cadherin, which was found to be induced in a dose-dependent manner in other experiments, instead as demonstrated by quantitative real-time PCR experiments and western blot analysis (Garulli et al., 2014). Other epithelial protein markers lacking to be differently identified in

Table 2 Biologically relevant proteins for DM-treated cell characterization

Protein name	Entry ID	Ratio DM-treated/A17 cells
Epithelial markers		
Keratin, type I cytoskeletal 10	K1C10	0.96
Keratin, type I cytoskeletal 15	K1C15	1.05
Keratin, type II cytoskeletal 2 epidermal	K22E	1.31
Keratin, type II cytoskeletal 1	K2C1	1.04
Keratin, type II cytoskeletal 1b	K2C1B	1.03
Keratin, type II cytoskeletal 5	K2C5	1.04
Proteins interacting with epithelial markers		
Catenin beta-1	CTNB1	1.27
Catenin delta-1	CTND1	2.18
Spectrin alpha chain, brain	SPTA2	DM unique
Vinculin	VINC	3.35
Cytoskeleton reorganization and cell adhesion		
Alpha-actinin-1	ACTN1	2.74
CD9 antigen	CD9	DM unique
Catenin alpha-1	CTNA1	1.32
Filamin-A	FLNA	2.37
Zyxin	ZYX	2.94
Apical-basolateral polarity-related proteins		
Protein scribble homolog	SCRIB	DM unique
Epithelial-related proteins		
Ephrin-B1 1	EFNB1	DM unique
Ephrin type-A receptor 2	EPHA2	DM unique
Lactadherin	MFGM	33
Regulation and cell death		
Aminoacyl-tRNA synthase complex-interacting multifunctional protein 1	AIMP1	DM unique
Bcl-2 homologous antagonist/killer	BAK	DM unique
Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit DAD1	DAD1	DM unique
Sphingosine-1-phosphate lyase 1	SGPL1	DM unique

Proteins characterizing DM-treated cells identified in the experiments are reported and divided as per functions. For each protein, the last column specifies the ratio of DM-treated to A17 cells (measured as spectral counting) or if the protein is DM treated unique

the experiment were ZO-1, laminin-1, claudins, occludins, or desmoplakin, and only keratins represented an exception (Kalluri and Weinberg, 2009; Thiery and Sleeman, 2006). In this experiment, different types of keratins have been identified in both cell samples and are summarized in Table 2. Among them, those specifically connected to epithelial tissues were two, keratin, type I cytoskeletal 10, and keratin, type II cytoskeletal 1b.

Apart from keratins, proteins that interact specifically with epithelial markers have also been found in DM-treated cells, such as spectrin alpha chain, which interacts with

calmodulin, a typical epithelial marker in a calcium-dependent movement of the cytoskeleton at the membrane. On the other hand, proteins that interact with E-cadherin were three. One of these proteins was CTNB1, which was identified in the DM-treated samples and which is usually present in complexes with E-cadherin and CTNA1 located to adherens junctions, which are typical components of epithelial cells. The second protein was catenin delta-1 (CTND1), involved in cell adhesion with cadherins, but also in ligand-induced receptor and Wnt signaling. What was significantly relevant about this protein is that it is part of a multiprotein cell–cell adhesion complexes in which E-cadherin, CTNA1, CTNB1, and gamma-catenin are also part. Vinculin was the last protein to be related to an epithelial marker: it is involved in cell-matrix adhesion and cell–cell adhesion regulating cell-surface E-cadherin expression; moreover, it is found in cell–cell junctions, which are structures typical of epithelial cells.

The presence of overexpressed proteins involved with cytoskeleton reorganization was another aspect that emerged from the GO data analysis about DM-treated cells. Relevant ones were actins, in particular alpha-actinin-1, an F-actin cross-linking protein belonging to the alpha-actin family that is thought to anchor actin to intracellular structures in a bundling function. This actin cytoskeleton reorganization could be interpreted as a progressive loss of mesenchymal features in favor of different, and more epithelial-like, type of organization. This aspect, together with the presence of proteins connected with cell junctions, also supported the mesenchymal to epithelial transition. These variations of the proteomic profile of DM-treated A17 cells were consistent with the morphological transition of A17 cells already observed in a previous study, where DM treatment induced the loss of A17 elongated mesenchymal phenotype to acquire a polygonal epithelial-like shape (Garulli et al., 2014). Other proteins connected with cell morphology were four: filamin-A, which was found overexpressed in DM-treated cells; CTNA1, which like other catenins forms complexes with the cytoplasmic domain of both E-cadherins and N-cadherins linking the actin filament network, seems to be important for cell-adhesion and it has been found in both samples; zyxin (ZYN), an adhesion plaque protein important for focal adhesion and for the formation of actin-rich structures; and CD9 antigen (CD9), DM-treated cell unique protein with several cellular processes, in particular it regulates paranodal junction formation and is involved in cell adhesion, cell motility, and tumor metastasis.

Another difference that could be correlated to a mesenchymal to epithelial transition upon DM treatment of A17 cells was the establishment of an apical-basolateral polarity, essential for the typical cell–cell interaction of

epithelial cells. In this sense it was interesting to observe the identification of protein scribble homolog, a protein involved in differentiation of epithelial and neuronal cells and responsible for the establishment of apical-basolateral cell polarity. This was peculiarly important because it has been found exclusively in DM-treated samples and could be consistent with a mesenchymal to epithelial transition.

According to GO data, other epithelial-related proteins could be found in the lists obtained in this experiment, in particular two membrane receptors have been found in the unique proteins, namely, ephrin-B1 and ephrin type-A receptor 2.

Another interesting feature to highlight was that DM-treated cells showed few proteins connected with cell growth, in particular with regulation and programmed cell death, which was in contrast with the mesenchymal feature of stem cell similarity. Among the proteins exclusively found in DM-treated cells possessing this feature, according to GO data, four proteins had a regulative effect upon apoptosis, i.e., bcl-2 homologous antagonist/killer, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit DAD1, sphingosine-1-phosphate lyase 1, and aminoacyl-tRNA synthase complex-interacting multifunctional protein 1.

Conclusions

Shotgun proteomics was used to investigate the BMP signaling pathway inhibition in cancer stem cells using A17 cells, as model A17 proteome profile showed a significant accordance with the expected mesenchymal phenotype from genomic investigations and also with more recent results obtained with different techniques (Garulli et al., 2014). Moreover, the additional GO search also helped in identifying additional biologically significant proteins that relate with mesenchymal features. The effect of DM treatment on these cells has also been evaluated, in particular taking into consideration a mesenchymal to epithelial transition. The analysis of GO data for DM-treated cells led to the conclusion that important changes have begun and a transition from mesenchymal-cell type to epithelial-cell type was at work at the end of a 3-day-long DM treatment. In fact, several mesenchymal markers, particularly adhesion molecules fibronectin, N-cadherin, vimentin, and several related proteins, were found to be absent or significantly depleted in DM-treated cells, suggesting that DM-treated cells acquired a metastable cell phenotype, i.e., they showed hybrid features in which both epithelial and mesenchymal traits were still kept. Consistent with the interpretation that the changes observed arise from epithelial specification from mesenchyme, a number of epithelial markers were observed to increase or to be expressed exclusively in the

DM-treated cells, including vinculin, E-cadherin, as well as an apical-basolateral polarity determining protein SCRIB. These results further supported, with an independent investigation technique, the mesenchymal epithelial transition already suggested for this system, also providing additional data from membrane protein expression and related GO data. Taken together, these results shed light on the key role of BMP signaling pathway in the maintenance of the mesenchymal phenotype in breast cancer, which could suggest BMP deprivation as a possible route for suppressing cancer stem cell ability to self renew and cause tumor recurrence, opening new therapy targets to fight cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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