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Proteomic Profiling of *In-Vitro* Bone-Conditioned SKBR3 Breast Cancer Cells

Ida Pucci-Minafra[1*], Gianluca Di Cara[1], Rosa Musso[1], Giovanni Peri[2], Biagio Valentino[2], Michele D'Arienzo[3], Desiree Martini[4], Mario Raspanti[5] and Salvatore Minafra[1]

[1]*Center of Experimental Oncobiology (C.OB.S), University of Palermo and La Maddalena Hospital, Palermo, Italy*
[2]*Department of Experimental Biomedicine and Clinical Neurosciences (BIONEC), University of Palermo, Italy*
[3]*Surgical Disciplines Department , Oncology and Dentistry (DICHIRONS), University of Palermo, Italy*
[4]*Department of Biomedical Sciences and Neuromotor (DIBINEM), sez Anatomia Umana, University of Bologna, Italy*
[5]*Department of Surgical Sciences and Morphological (DSCM), University of Insubria, Varese, Italy*

Abstract

A major complication of advanced breast cancer is the frequent formation of bone metastases. To our knowledge, however, the information on the phenotypic properties of cancer cells able to survive and colonize the bone tissue is still fragmentary. We report here a proteomic study of breast cancer cells (SKBR3) collected after a colonization of bone fragments in a special model of co-culture, used to simulate the phenotype of breast cancer metastasizing cells. The scanning electron microscopy of the seeded cancer cells into the bone fragments, showed their peculiar traits of multilayered growth and spiny surface, typical of a very aggressive phenotype. The comparative proteomic profiling of the bone-conditioned cells (SKBR3-B1) versus the parental cell line (SKBR3-WT), performed by applying two-dimensional electrophoresis (2D-IPG) and mass spectrometry (MALDI-TOF) revealed the existence of an interesting differential proteomic profiling. Within this pattern, an increase of several members of the S100 protein family, not described before in relation to bone metastasis from breast cancer, was observed in the SKBR3-B1. Other up-regulated proteins belong to protein classes involved mainly in cytoskeleton dynamics, chaperone / folding, negative regulation of apoptosis and cell metabolism. These results suggest that, rather than individual proteins, a coordinated network of strategic proteins is able to determine or drive the invasive and metastatic phenotype of breast cancer cells, namely: proteins primarily strategic for the cell penetration and homing into the bone, and proteins coming into play to satisfy the modified metabolic needs of the cells. We believe that the present study represents an innovative approach of co-culture with fresh human bone fragments, and that it may contribute greatly to improve the knowledge of the molecular mechanisms underlying the propensity of breast cancer cells to colonize, survive and proliferate into the bone microenvironment.

Keywords: Breast cancer cells; Bone metastasis; Proteomics

Introduction

The majority of breast cancers (95% or more) are of epithelial origin, arising either from the ductal or from the lobular epithelium [1]. Through histopathological diagnosis both types can be distinguished as *in situ* or *invasive* carcinomas. The in-situ carcinomas grow within the inner border of the epithelium and are not aggressive, unlike the invasive ones that penetrate the tissue barriers and tend to form metastases. However, within these two main groups, histological and intra-tumoral heterogeneities have been identified and classified as breast carcinoma subtypes having different clinical outcomes and response to therapy.

Besides the classical histopathology, other methods to classify breast tumors are applied; these include molecular and genetic analysis, clinical pathology and gene expression profiling. However, at present, no adequate markers are available to depict a portrait of bone-metastatic breast cancer cells.

The metastatic process begins when the tumor cells of epithelial derivation detach from the original tissue, penetrate the basal lamina and invade the surrounding connective tissue to enter into the bloodstream or lymphatic vessels and reach distant anatomic sites [2]. To accomplish this program, the initially altered epithelial cells undergo significant changes, from a stationary polarized phenotype towards a mesenchymal migratory phenotype, a process known as "epithelial to mesenchymal transition, EMT" [3,4].

However, neither all the neoplastic cells from a primary tumor will go through the multistep metastatic process, nor all the metastatic cells will colonize tissues and organs to the same extent. In the case of breast cancer one of the most common sites is the bone [5].

Studies on *in vitro* and *in vivo* models have demonstrated the complexity of the whole process of bone metastasis, which goes from the osteotropism to the nesting in the bone where the cells lurk, proliferate and migrate out again into other areas of the bone or in adjacent spaces [6-10]. However, the phenotypic traits of cells homing towards the bone have not been adequately explored.

The objective of this work was to study the proteomic profiling expressed by breast cancer cells after their nesting into the bone tissue. With this aim, we have applied an *in vitro* system of breast cancer cells (SKBR3) cultured in the presence of fragments of bone obtained from healthy informed donors during reparative surgery.

Through comparative and subtractive proteomics between the

***Corresponding author:** Ida Pucci-Minafra, Center of Experimental Oncobiology (C.OB.S), University of Palermo and La Maddalena Hospital, Palermo, Italy, Tel: 39 3470471757; E-mail: pucci.ida@gmail.com

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SKBR3 before (SKBR3-WT) and after the *in vitro* invasion of the bone tissues (SKBR3-B1), we have derived a collection of overexpressed proteins, likely generating functional networks involved in the mechanisms of cell penetration, homing and survival into the bone.

Material and Methods

Cell culture

The SKBR3 cell line has been selected and the MCF10A cell line (ATCC, USA) was used as control for the present experiments. The SKBR3 is a breast cancer cell line derived from a pleural effusion of mammary adenocarcinoma and over-expressing HER2 / *c-erb-2* gene. Cells were grown in RPMI-1640 (EuroClone, Italy) with 10% Fetal Bovine Serum (FBS, EuroClone, Italy), 100 U/ml penicillin and 100 µg/ml streptomycin.

The MCF10A (BS CL 174) is a non-tumorigenic epithelial cell line, derived from a non-tumoral mammary gland. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, EuroClone, Italy) supplemented with: 10% FBS, 100 U/ml penicillin / 100 µg/ml streptomycin, 20 ng/ml EGF (Sigma-Aldrich, USA), 50 nmol/ml Hydrocortisone (Sigma-Aldrich, USA), 10 µg / ml Insulin (Sigma-Aldrich, USA) and 100 ng/ml Cholera Toxin (Sigma-Aldrich, USA). The cell lines were kept at 37°C in an atmosphere of 5% CO_2.

Both cell lines were recently submitted to a genetic test for their identity confirmation (BMR Genomics, Italy).

Bone fragments in cell culture assays

Bone fragments obtained as surgical waste from consenting informed donors were taken from tibial plates of 3 young patients (age 30-35), randomly selected among subjects operated on after traumatic events.

Soon after their arrival in the laboratory, the fragments were rinsed several times in sterile PBS and transferred in 6-well plates (EuroClone) with RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The wells with the bone fragments were kept in a 5% CO_2 incubator at 37°C for seven days to allow the release of weakly attached resident cells. Following this period, the fragments were relocated into new wells, where sub-confluent SKBR3 and MCF10A cells were seeded separately at the concentration of 15.000 / cm^2. Additional wells containing SKBR3 and MCF10A cells alone were used as controls.

Scanning electron microscopy

Several bone fragments co-cultured with SKBR3 cells and some fragments, not exposed to the neoplastic cell culture and used as controls, were rinsed with PBS and fixed with Karnovsky solution (1.5% glutaraldehyde, 1% paraformaldehyde, 1% cacodylate buffer, pH 7.4) for the SEM observation, performed at the Bologna and Varese laboratories. The bone fragments were then rinsed three times with 0.1% cacodylate buffer, postfixed for 20 min with 1% OsO_4 in cacodylate buffer, dehydrated with ethanol, and finally dried with hexamethyldisilazane (Sigma-Aldrich, USA) for 15 min, as described [11]. The specimens were sputter-coated with 15 nm of gold-palladium alloy system in a Balzers SCD-004 apparatus and then visualized with either a Philips 515 SEM operated at 15 kV or a FEI XL-30 FEG-SEM operated at 7 kV.

Immuno-assays

Western blotting: Following the electrophoretic runs on sodium dodecyl sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE), the gels of cell lysates were electrotransferred onto nitrocellulose membrane (HyBond ECL; Amersham) and stained with Ponceau S (Sigma Aldrich). The membranes were then probed with the following monoclonal (mAb) or polyclonal (pAb) antibodies: anti-vimentin (clone 3B4) mouse mAb (1:500; Novocastra); anti-cytokeratin 8 (C51) mouse mAb (1:500; Santa Cruz Biotechnology, SCBT); anti-cytokeratin 18 (DC-10) mouse mAb (1:500; Merck KGaA); anti-actin (Ab-1) mouse mAb (1:20000; Merck KGaA); anti-c-Myc (9E10) mouse mAb (1:500; SCBT); anti-Matrix Metallo Proteases: anti-MMP2 (8B4) mouse mAb (1:500; SCBT); anti-MMP9 (C-7) mouse mAb (1:500; SCBT); anti-MT-MMP1 (MM0027-9E10) mouse mAb (1:500; SCBT); anti-α Enolase (H-300) rabbit pAb (1:10000, SCBT); anti-galectin 1 (1E8-1B2) mouse mAb (1:1000, Novus Biological); anti-glyceraldheyde-3-phospate dehydrogenase (0411) mouse mAb (1:1000, SCBT); anti-S100A7 (47C1068) mouse mAb (1:1000, SCBT).

Following incubation with the appropriate peroxidase-linked antibodies the reaction was revealed by the Enhanced chemiluminescence (ECL) detection system, using high performance films (Hyperfilm ECL; Amersham). Secondary antibodies were the following: horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000; GE Healthcare Europe GmbH); goat anti-mouse IgM, H&L chain specific peroxidase conjugate (1:10000; Merck KGaA).

Immunocytochemistry (ICC): SKBR3 cells were grown on slides until sub-confluent (four days from seeding) and then fixed using methanol-based buffered preservative solution (ThinPrep PreservCyt Solution) according to the manufacturer's instructions.

ICC analysis was carried out using the anti-Neu (300G9) mouse mAb (1:100; Santa Cruz). Incubation with primary antibody was carried out at 37°C for 30 minutes followed by washing with Phosphate Buffered Saline (PBS) solution (EuroClone). The reaction was carried out with the ImmunoCruz™ mouse ABC Staining System. The cells were counterstained with haematoxylin-eosin (Sigma). Negative controls were performed by excluding the primary antibody.

Two dimensional gel electrophoresis

Protein extracted from cell lysates were submitted to extensive dialysis against ultrapure distilled water at 4°C and lyophilized. Dried samples were solubilized in a buffer containing 4% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) (Sigma-Aldrich, USA), 40 mM Trizma base (Sigma-Aldrich, USA), 65 mM DTE (1,4-Dithioerythritol) (Sigma-Aldrich, USA) and a trace of bromophenol blue in 8 M urea. Aliquots of 45 µg (analytical gels) or 1.5 mg (preparative gels) of total proteins (Bradford assay) were separately mixed with 350 µl of rehydration solution containing 8 M urea, 2% CHAPS, 10 mM DTE and 0.5% carrier ampholytes (Resolyte 3.5-10; Amersham), and applied for isoelectrofocusing (IEF) using commercial sigmoidal immobilized pH gradients (IPG) strips, 18 cm long with pH range 3.0-10 (Bio-Rad, USA). Isoelectrofocusing conditions were from 200 to 3500 V (3 hrs), 8000 V (8 hrs). The focused proteins were then separated on 9–16% linear gradient polyacrylamide gels (SDS-PAGE) with a constant current of 20 mA / gel at 10°C and the separated proteins were visualized by ammoniacal silver staining [12].

Silver-stained gels were digitized using a computing densitometer and analyzed with ImageMaster 2D PLATINUM software (Amersham Biosciences). Gel calibration was carried out using an internal standard

and the support of the ExPaSy molecular biology server, as described [13].

Protein identification

In-gel digestion and MALDI-TOF analysis: Mass spectrometry was performed with the Voyager DE-PRO (Applied Biosystems) mass spectrometer as described [14]. Proteins were digested using sequencing-grade trypsin 20 µg / vial), and the peptides were re-dissolved in 10 µl of 0.1% trifluoroacetic acid (TFA) and spotted in HCCA (R-cyano-4-hydroxycinnamic acid) matrix (Sigma-Aldrich). MALDI-TOF mass spectra were recorded in the 500-5000 Da mass range, using a minimum of 150 shots of laser per spectrum. Internal calibration was carried out using trypsin autolysis fragments at m/z 842.5100, 1045.5642, and 2211.1046 Da. Peptide mass fingerprinting was compared to the theoretical masses from the Swiss-Prot or NCBI sequence databases using Mascot (http://www.matrixscience.com/). Typical search parameters were as follows: 50 ppm of mass tolerance, carbamidomethylation of cysteine residues, one missed enzymatic cleavage for trypsin, a minimum of four peptide mass hits was required for a match, methionine residues could be considered in an oxidized form.

In order to support the reliability of the spot identification, 2D-based Western Blot immuno-detection was applied to a selection of 5 mass-detected proteins, 3 of which are house-keeping, actin, glyceraldehide-3phospate and enolase, and 2 are regulated proteins, galectin and S10A7. Figure 1 shows a perfect coincidence of the two sets of profiles.

Results

Cell culture with bone fragments

Bone fragments obtained and prepared as described in the Material and Methods section, were located into wells where sub-confluent SKBR3 cells were seeded at the concentration of 15.000/cm^2. Figure 2a shows the SKBR3 cell culture at sub-confluence. In the inset the strong immune-cytochemical reactions of the SKBR3 with the ERBB2 / Neu antibody is shown, testifying the amplification of this gene expression in the cell line. The non-tumoral epithelial mammary cells, MCF10A, were placed in culture with the bone fragments, as a control experiment.

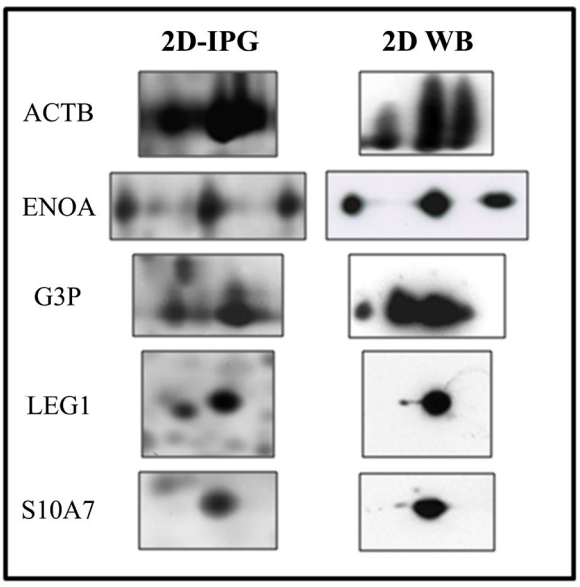

Figure 1: Panel of western blot assays performed on selected proteins separated by 2D-IPG, to validate the Maldi / TOF identification procedure. ACTB (Actin B), ENOA (alpha-Enolase), G3P (Glyceraldheyde 3P dehydrogenase), LEG1 (Galectin 1), S10A7 (Psoriasin)

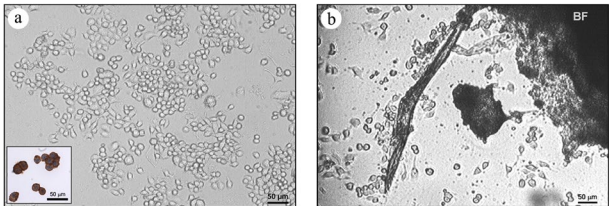

Figure 2: a) SKBR3 cell culture at sub-confluence, showing the apolarized cell morphology, typical of neoplastic cells. The inset shows the strong immune-cytochemical reaction of the SKBR3 with the ERBB2 / Neu antibody, testifying the amplification of this gene expression in the cell line; b) A spontaneous tropism of SKRB3 cells towards the edge of the bone fragments (BF) to which cells adhere.

The culture of cells with bone fragments were monitored daily. After four days an apparent spontaneous tropism of SKRB3 cells was observed towards the edge of the bone fragments to which cells adhere (Figure 2b). The cell culture was continued until the time of the cell confluence (7th day). No tropic activity was observed for the MCF10A cells (not shown).

The bone fragments placed in culture with SKBR3 cells were then collected in two separated sets and submitted to two different experimental approaches: one set was fixed and prepared for the scanning electron microscopy; the second was placed in a new well with fresh RPMI culture medium in the CO_2 incubator at 37°C and monitored daily.

A parallel set of bone fragments not exposed to the neoplastic cell culture, was submitted to the scanning electron microscope observation.

Scanning electron microscopy

The results obtained with the SEM highlighted the presence of colonies of cells in the bone fragments co-cultured with the SKBR3 cells. Figure 3a shows an expanding cell colony growing within a bone matrix niche. Typically, the cells showed a rather uniform morphology and were grouped closely to each other, still maintaining a roundish outline with a very spiny cell surface. At a higher magnification the cell surface displayed the emission of irregular membrane ruffling and protrusions by which cells appear to cling tenaciously onto the bone tissue. A vesiculation activity, typical of malignant cancer cells with motile phenotype, also appeared (Figures 3b and 3c). Figure 3d reports the scanning micrograph of a control bone fragment showing the basic structure of the bone scaffold.

Cell sprouting from bone fragments

As already mentioned the second set of co-cultured fragments was placed into new wells and monitored daily. After 4 days an out-growth of cells was observed. Figures 4a and 4b show the progressive growth of colonies of cells, which, as being derived from a bone colonization, are named SKBR3-B1 (where B1 stands for "Bone-Conditioned") to be distinguished from the parental cells named SKBR3-WT. By monitoring the time dependent outgrowth of SKBR3-B1 cells from the bone fragments we observed that the dynamic and modality of the bone

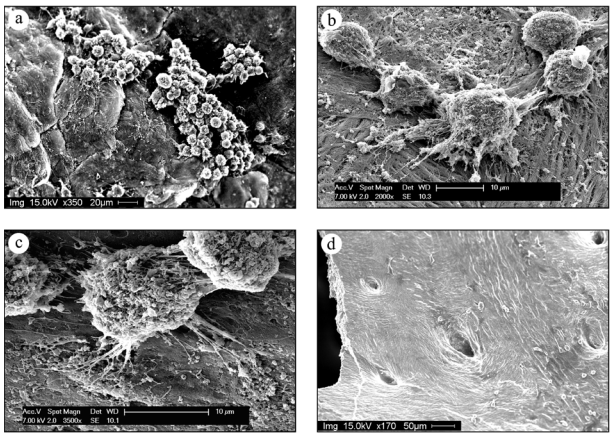

Figure 3: a) SEM micrograph of SKBR3 cell colonies growing within niches on a bone matrix fragment. The cells are growing in tight clusters and maintain a rounded shape; b) Detail of a small cluster of the SKBR3 breast cancer cells within the bone fragment; c) Higher magnification of the SKBR3 cell surface showing the elongated projections adhering to the bone matrix and producing an active vesiculation. The cells show numerous elongated cytoplasmic projections by which they establish reciprocal contacts and remain adherent to the bone; d) Low magnification SEM picture of a control bone fragment, showing the basic structure of the bone scaffold.

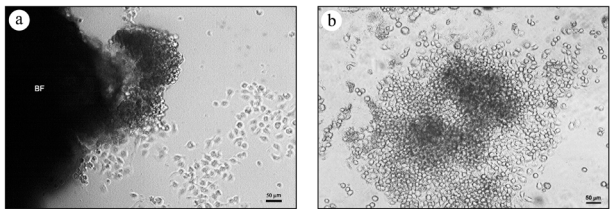

Figure 4: a) Outgrowth of the SKBR3 cells from the bone fragment (BF) in which they penetrated following the co-culture experiments. The new cell population sprouting from the bone fragments is named SKBR3–B1, where B1 stands for "bone conditioned"; b) The progressive three-dimensional overgrowth of SKBR3-B1 cell colonies derived from the bone colonization.

conditioned cell-growth appeared typical for very aggressive neoplastic cells: as can be seen in Figure 4b, cells grow three-dimensionally one over the other, from the initial sprouting, and form multilayered colonies, independent of cell density and space availability, typical of the very aggressive cancer cells.

At fifteen days of co-culture, the cells released from the bone fragments were properly collected and subjected to protein extraction for the proteomic evaluations.

Immunochemical assessment

The phenotypic nature of the cells released from the bone fragments was further assessed by western blot using cytokeratins 8 and 18 (as epithelial markers) vimentin (mesenchymal marker, related to the epithelial-mesenchymal transition in cancer cells), cMyc oncogene to assess the cancer phenotype of these cells. The result of the assays revealed high expression levels of these proteins, confirming the epithelial origin and the tumoral phenotype of the SKBR3-B1 cells (Figure 5). To verify the potential collagenolytic capacity of the SKBR3-B1 cells, a battery of antibodies against most MMPs involved in the degradation of the extracellular matrix (MMP1, MMP2, MMP3, MMP9, MMP14) was also tested. As shown in Figure 5, strong positivity was obtained for the MMP1 in the SKBR3-B1 cells, in contrast to the SKBR3-WT, while faint or absent signals was obtained by the other MMPs antibodies (not shown).

Proteomic profiling

Bone-conditioned cells, SKBR3-B1 were collected and prepared for the proteomic assays in parallel with the parental cell line, SKBR3-WT. Figure 6 shows a prototype of 2D-IPG reference map of the SKBR3-WT, where 373 protein spots were identified by the MALDI-TOF procedure immunologically validated by Western blot with selected antibodies, as reported in the MM section (see Figure 1). Supplementary Table 1 reports the list of identified proteins with the associated parameters.

The identified proteins were grouped into 13 functional clusters (Supplementary Table 2), on the basis of their primary function or subcellular localization according mainly to David Bioinformatics Resources [15,16] namely: Calcium Binding; Chaperones / Folding and Vesiculation; Cytoskeleton; Cytoskeleton Organization and Cell

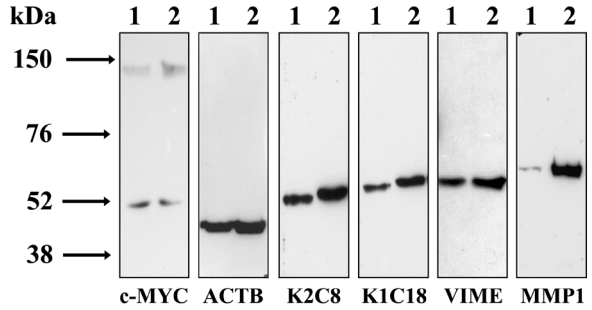

Figure 5: Panel of western blot assays performed on SKBR3-WT (lane1) and SKBR3-B1 (lane2) respectively, with following antibodies: anti-c-Myc, anti-ACTB, anti-K2C8, anti-K1C18, anti-VIME, and anti-MMP1.

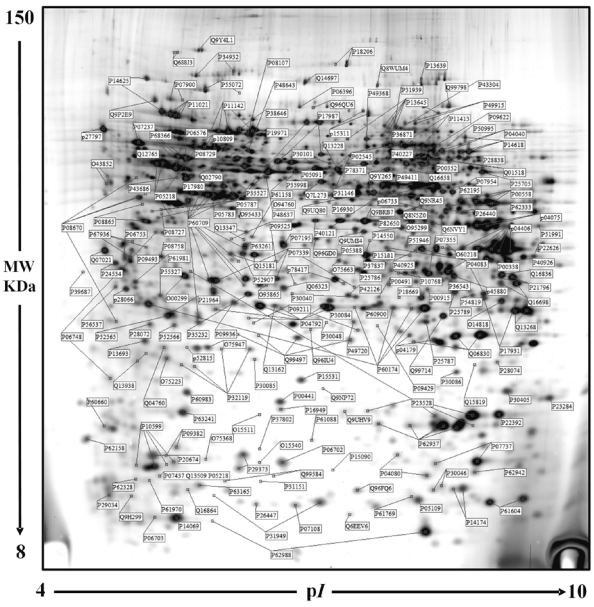

Figure 6: Miniature of the reference MAP of SKBR3-WT. The 373 identified protein spots are labeled with the Uniprot AC number. When present, different isoforms of the same protein are jointly labeled.

Motility; Detoxification; Ionic Homeostasis; Metabolic Enzymes; Mitochondrial Proteins; Nuclear Proteins; Nucleoside-related Processes; Processing of Metabolites and Aminoacids; Protein with Extracellular Activities; Protein Biosynthesis, Degradation and Modulation.

Finally, a "transverse" class of proteins belonging to different functional classes, but sharing an anti apoptotic activity *(*Negative regulation of apoptosis*)* was created.

Comparative profiling of SKBR3-B1 and SKBR3-WT: For the comparative profiling between the two cell cultures, the quantitative analysis of protein spots was performed in duplicate maps, and normalized as % vol (integration of optical density over the spot area) to correct for staining variations. The number of differentially expressed protein spots (including isoforms) displaying an increase in the intensity values greater than 50% was of 63 corresponding to 17% of the total proteomic complement. These data are presented in the histograms in Figure 7.

The most represented proteins, among the overexpressed, belong to the following categories: Calcium Binding (12,7%), Chaperones / Folding and Vesiculation (14%), Cytoskeleton (9,5%), Organization and Cell Motility (11%), Detoxification (11%) and Protein Biosynthesis, Degradation and Modulation (14%).

In addition, it was observed that 15 out of 63 overexpressed proteins (including isoforms) belonging to the above functional clusters, exerted anti-apoptotic activity.

Discussion

The major problem in cancer treatment is the formation of metastases for which there is still no adequate diagnostic and prognostic approach. In particular, bone metastases are the most insidious and risky type of metastasis, because of the nature of the host tissue, their rapid spreading inside and outside of the tissue, the difficulty of treatment, and the debilitating consequences and the frequent lethal progression

In this study we set up a special model of co-culture to simulate the phenotype of breast cancer metastasizing cells, using bone fragments from healthy subjects operated on for trauma, and the breast cancer-derived cell line, SKBR3. As a negative control the non-tumoral cell line MCF10 was used.

The scanning electron microscopy has revealed that the SKBR3 cells, contrary to the MCF10A cells, were able to penetrate into the bone fragments digging small niches and producing colonies. These cells, named SKBR3-B1, were collected and further analyzed.

Morphological and western blot immuno-assays testified the epithelial origin of these cells; their neoplastic nature was confirmed by the modality of multi-layered growth, by the expression of two typical breast cancer oncogenes, ERBB2 and c-Myc, while the production of proteins which are typical expressions of mesenchymal cells, as vimentin and MMP1, gave evidence of their EMT transition.

The comparative proteomics profiles between the SKBR3-B1 cells and the parental cells (SKBR3-WT), revealed that the bone-conditioned

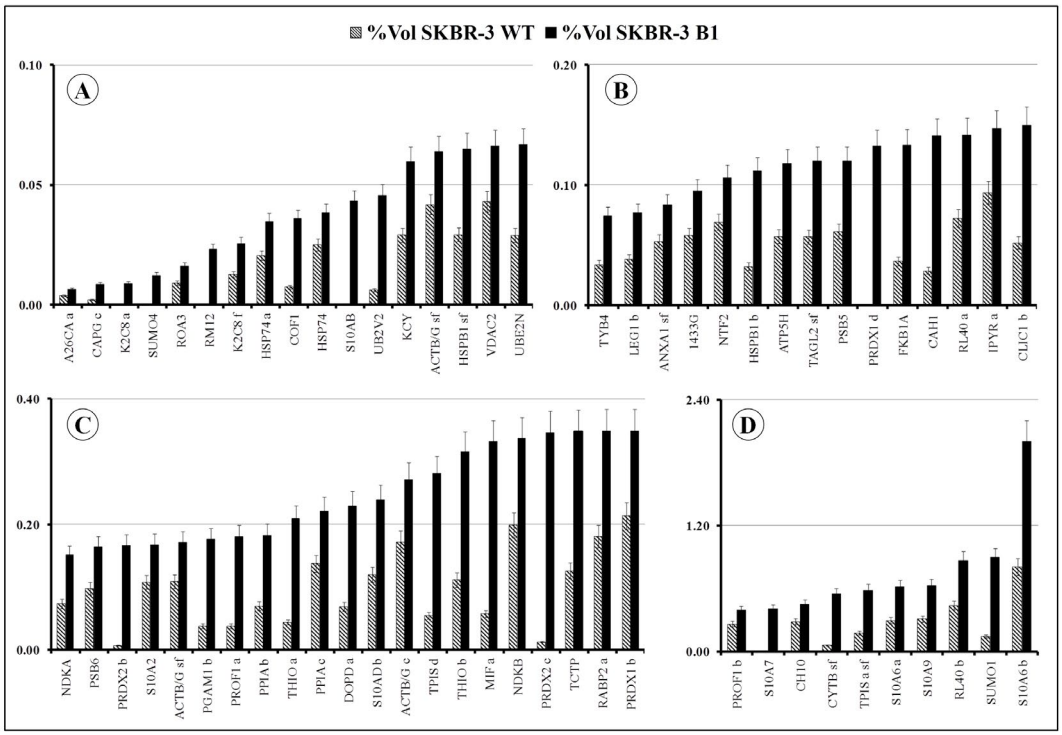

Figure 7: Comparative histograms of the differentially expressed protein spots displaying an increase in the intensity values greater than 50% in SKBR3-B1 proteins *versus* the SKBR3-WT. Each spot value is the average of 2 separate duplicated assays. Standard deviation is given as vertical error bars. Proteins are indicated by abbreviated names to UniProtKB database. In the ordinate are the % volumes of the protein spots exported from ImageMaster software. Histograms are grouped by intensity ranges (panels a, b, c and d).

cells displayed an overexpression (cut off 50%) of significant proteins commented below.

Calcium binding

This group includes Annexins and S100 proteins. The S100 family is the largest group of EF-hand calcium binding proteins with signaling properties. To date, at least 25 distinct members of this family have been recognized in humans. Mostly, they are expressed in a tissue-specific manner and may perform different intracellular and extracellular functions involved in several biological and pathological processes including cancer [17-20].

In addition, many of the S100 proteins are involved in the carcinogenesis by participating in the process of epithelial-mesenchymal transition [21], activation of cell migration [22] and the induction of the expression of metalloproteinases related to the invasive phase of the tumor [23]. The most representative members of the S100 family in the bone conditioned cells were the following: S10A2, S10A6, S10A7, S10A9, S10AB, S10AD.

It has been reported that S10A2 interacts with p53 so modulating its transcriptional activity [24]. Moreover S10A2 has been shown to interact with Smad3, to regulate TGF-β / Smad3 signaling and to induce EMT in lung cancer [25]. Our results show that the increase of S10A2 is also concomitant with the enhanced MMP1 protein expression, which may be instrumental for the collagenolysis required for the neoplastic cells to home onto the bone fragments. At present, no data have been reported on the role of S10A2 in bone metastasis.

The S10A6 has been demonstrated to be involved in many types of cancers and linked to metastasis. Recent studies in patients with pancreatic cancer have demonstrated a strong link between high expression of nuclear S10A6 and poor survival. While, depletion of S10A6 has been shown to exert a strong effect on the reversion of invasion and motility of pancreatic cancer cells [26]. No information is available yet on the possible role of this protein in the bone metastasis of cancer cells.

Concerning the S10A7, our previous study on a collection of breast cancer surgical fragments demonstrated that this protein is sporadically present in breast cancer patients with the same clinical diagnosis, but when present it was highly expressed both in the cytoplasm and in the nucleus of the neoplastic cells, and absent from the surrounding tissues [27]. Other Authors reported that S10A7 increases invasive capabilities of prostate cancer cells via a regulation of MMPs [28]. Another report shows that S10A7 inhibits β-catenin signaling by promoting β-catenin degradation. In turn, β-catenin signaling negatively regulates S10A7 expression. Thus, the reciprocal negative regulation contributes to their important roles in tumor progression [29].

Regarding S10A9 it is known that it localizes with its partner S10A8 in several biological systems but it may act as an individual player in many cancers [30].

It is believed that the expression of S10A9 correlates with inflammation, cancer or other pathological processes. In gastric cancer S10A8 / A9 have been shown to promote cell migration and invasion through an increase of MMP2 and MMP12 via p38 MAPK dependent NF-κB activation [31].

Other reports indicate that S10AB and S10AD are correlated, respectively, with the progression of pancreatic adenocarcinomas [32] and with the *in vitro* invasivity of lung cancer cells [33]. It has also been reported that S10AB is required for efficient plasma membrane repair and survival, necessary for the metastatic spreading of invasive cancer cells [34].

Interestingly, among the targets of these signals are the p53 activator of cJun which is co-promoter of MMP1 transcription. In addition another protein overexpressed in SKBR3-B1, SUMO1 (included in the group of Protein Biosynthesis, Degradation and Modulation), has been shown to modulate c-Jun and p53 activity [35].

Chaperones / folding and vesiculation

It is known that many stimuli, including oxidative stress and heavy metals, can activate stress responses in cells, besides the originally documented temperature shock. Major effects of cellular stress are protein aggregation and misfolding. A typical cell defense is the increase of heat shock / chaperon expression that helps in the protein refolding and disaggregation. In addition, these proteins may play essential roles in tumor growth, either by promoting autonomous cell proliferation or by inhibiting cell death pathways [36]. Alterations in HSPs expression are known to affect cell behavior including self-renewal, differentiation, sensitivity to environmental stress and aging [37]. Many of the HSPs are expressed at high levels in a wide range of tumors, where their expression is correlated with a poor prognosis and resistance to therapy. The increased transcription of HSPs in tumor cells is thought to be due to the loss of p53 function and to a higher expression of the proto-oncogenes HER2 and c-Myc [38]. Both oncogenes are found overexpressed in the SKBR3 cells. Numerous reports have indicated that some HSPs physically interact with several transcription factors and cofactors through intrinsic and extrinsic signaling pathways.

Mammalian HSPs have been classified into several families according to their molecular weight: HSP100, HSP90, HSP72, and small molecular HSPs (including HSPB1). Among the proteins of these families, the ones significantly over-expressed in the SKBR3-B1 with respect to the MCF10A cells and increased in respect to the SKBR3-WT, are the following proteins or isforms: CH10, HSPB1 (also known as HSP27), HSP74.

CH10 is a 10 kDa, highly conserved, mitochondrion-resident protein, which co-chaperones with another mitochondrial heat shock protein, the CH60. Eukaryotic CH10, with its partner CH60, is essential for mitochondrial protein biogenesis, as well as for the assembly and disassembly of protein complexes [39]. In addition, it has been well documented that the expression of small HSPs, especially HSPB1 and the inducible HSP70, enhances the survival of mammalian cells exposed to numerous types of stimuli that induce stress and apoptosis [36]. HSPB1 and HSP70 with anti-apoptotic roles are abundantly expressed in many malignant human tumors [40].

Other proteins with chaperone (RABP2) and folding (FKB1A, PPIA) activities were also differentially expressed in the SKBR3-B1 cells, but to a lower extent with respect to the HSPs already mentioned.

Cytoskeleton organization and cell motility

Among the overexpressed proteins in the bone conditioned cells, the proteins involved in the cytoskeleton reorganization deserve particular attention. The reorganization of cytoskeleton is an early cellular response to a variety of extracellular signals and is involved in essential cell functions such as: membrane trafficking, motility, retaining cell shape and polarity, cell proliferation and survival. PROF1 and COF1 are actin-binding proteins which play a crucial role in the turnover and restructuring of the actin cytoskeleton and so controlling

several cellular processes. PROF1 has been found to be involved in lipid based signaling and in the regulation of glycolysis through the axis of G protein subunit, Gα13 / EGR1 [41]. COF1 has been found to be involved in several cell processes, among which the initiation phase of apoptosis [42]. It has also been shown to contribute to the contact inhibition and proliferation in cancer, through YAP / TAZ activity [43] and to import actin monomers into the nucleus under certain stress conditions. Recent studies propose the cofilin pathway as a major determinant of metastasis [44].

TYB4 is a regulator of actin assembly by its ability of depolymerization of actin filaments. It has been reported that TYB4 increases cell migration, angiogenesis and tumor metastasis of colon cancers [45].

It is reasonable to believe that even in bone metastases the cytoskeleton-associated proteins mentioned above play an important role in the migration and in the homing into the bone of the osteotropic cells.

Another over-expressed protein with multiple functions, including cytoskeleton dynamics, is the macrophage inhibitor factor, MIF, which may function either intracellularly or as a secreted cytokine. It is over-expressed in many solid tumors and is associated with poor prognosis.

The intracellular actions of MIF include the binding of Jab1, which is a co-activator of AP-1 transcription that also promotes the activation of MMPs transcription [46].

As extracellular cytokine, MIF is an important regulator of innate immunity, but it also participates in the promotion of tumor growth and metastasis [47].

Finally, CAPG and STMN1 over-expressed in SKBR3-B1, exert profound influences on cell proliferation, differentiation and cell motility in response to specific signals. All features characterize tumor growth and invasive behavior [48,49].

Detoxification

The detoxification proteins can be mainly classified into Phase I enzymes, Phase II enzymes and antioxidant enzymes according to their functional mechanisms (cfr. DetoxiProt database). Collectively these proteins can partially eliminate the active oxygen species produced by exogenous or endogenous toxins. In the bone conditioned cells the identified detoxification proteins which appeared over-expressed, include CLIC1, PRDX1, PRDX2, THIO.

Some of them, i.e. THIO can play additional functions including the control of cellular growth and apoptosis, and the modulation a number of extracellular inflammatory processes [50].

Protein biosynthesis, degradation and modulation

Among these protein groups, it is worth mentioning the small ubiquitin-related modifier, SUMO. In mammals, four different genetic isoforms, termed SUMO-1,-2,-3 and -4 have been identified so far. SUMO proteins are critically involved in the modulation of nuclear organization and cell viability. Their expression is significantly increased in processes associated with carcinogenesis, such as: cell growth, differentiation, senescence, oxidative stress and apoptosis. In our system we found over-expression of SUMO1 and SUMO4. SUMO1 plays a crucial role as a transcriptional co-regulation in various cellular pathways, including the p53 pathway in colon cancer [51] and HIF1α in prostate cancer [52]. Among the final targets of these pathways is the promoter of some metalloprotease genes, namely MMP1, MMP2 and MMP9, through direct or indirect pathways [53]. SUMO4, upon oxidative stress, conjugates to various anti-oxidant enzymes, chaperones, and stress defence proteins. It may also conjugate to some transcriptional regulators, positively or negatively regulating their activities. It has been shown that SUMO 4 may conjugate SUMO1 through SENP1 (sumo specific protease 1) and JUN therefore contributing to the modulation of the key enzyme of the invasion and metastasis, MMP1, MMP2 and MMP9 [54].

Negative regulation of apoptosis

Finally it is worth noting the observation that 24% of the protein over-expressed (15 out of 63, including isoforms), among the different functional classes have in common an antiapoptotic role. These are: COF1, MIF (Cytoskeleton, Organization and Cell Motility), NDKA, NDKB (Nucleoside-related proteins), CYTB (Nuclear proteins), RL40 (Protein Biosynthesis, Degradation and Modulation), PRDX1, PRDX2 (Detoxification), CH10, HSPB1 (Chaperones, Folding and Vesiculation), TCTP (Ionic Homeostasis).

Conclusion

In conclusion, the clusterization of proteins detected in this study, shows that the complex pattern of proteins over-expressed in the SKBR3-B1 cells can be subdivided into two major key groups: a) proteins primarily strategic for the cell penetration and homing into the bone, and b) proteins functioning as co-actors, which come into play to meet the modified metabolic needs of the cells.

The first group includes proteins of the calcium-binding family, the matrix metalloprotease. MMP1, proteins with anti-apoptotic activities and proteins of cell motility. The synergy among these proteins is reasonably required for bone demineralization and local digestion of collagen fibers to dig the niche where the cells home and proliferate. The anti-apoptotic proteins are instrumental for the continuation of cell proliferation and the formation of colonies, while the cell matrix adhesion, clearly observed in scanning micrograph, is likely to be facilitated by the proteins of the cell motility group, involved in the formation of membrane protrusions.

The second group includes proteins of metabolism, heat shock / chaperon and other proteins involved in the multiplicity of metabolic and basic processes of cell life.

Conclusively, we suggest that not only a single member within these groups of overexpressed proteins, but the inter-connected activity of the majority of them, is able to determine or drive the invasive and metastatic phenotype of breast cancer cells.

We believe that this present work represents a strong innovative effort due primarily to the co-culture system with fresh human healthy bone fragments and breast cancer cells. Indeed to our knowledge no functional proteomic study on bone metastasis utilizing human tissue fragments has been reported in literature while, several reports have been produced on animal models [55]. These results may contribute to the growing knowledge of the breast cancer proteomic atlas, and to the identification of new therapeutic targets for future molecular therapies against metastatic breast cancer.

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References

1. Tomaskovic-Crook E, Thompson EW, Thiery JP (2009) Epithelial to mesenchymal transition and breast cancer. Breast Cancer Res 11: 213.
2. Pucci Minafra I (2012) Extracellular matrix in breast cancer: permissive and restrictive influences emanating from the stroma. In: Karamanos N (Ed) Extracellular Matrix: Pathobiology and Signaling. Walter de Gruyter GmbH, Inc., Berlin.
3. Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428.
4. Minafra L, Bravatà V, Forte GI, Cammarata FP, Gilardi MC, et al. (2014) Gene expression profiling of epithelial-mesenchymal transition in primary breast cancer cell culture. Anticancer Res 34: 2173-2183.
5. Weidle UH, Birzele F, Kollmorgen G, Rüger R (2016) Molecular Mechanisms of Bone Metastasis. Cancer Genomics Proteomics 13: 1-12.
6. Holen I, Nutter F, Wilkinson JM, Evans CA, Avgoustou P, et al. (2015) Human breast cancer bone metastasis *in vitro* and *in vivo*: a novel 3D model system for studies of tumour cell-bone cell interactions. Clin Exp Metastasis 32: 689-702.
7. Sloan EK, Anderson RL (2002) Genes involved in breast cancer metastasis to bone. Cell Mol Life Sci 59: 1491-1502.
8. Rucci N, Teti A (2010) Osteomimicry: how tumor cells try to deceive the bone. Front Biosci (Schol Ed) 2: 907-915.
9. Theriault RL, Theriault RL (2012) Biology of bone metastases. Cancer Control 19: 92-101.
10. Kingsley LA, Fournier PG, Chirgwin JM, Guise TA (2007) Molecular biology of bone metastasis. Mol Cancer Ther 6: 2609-2617.
11. Pucci-Minafra I, Cancemi P, Di Cara G, Minafra L, Feo S, et al. (2008) Decorin transfection induces proteomic and phenotypic modulation in breast cancer cells 8701-BC. Connect Tissue Res 49: 30-41.
12. Pucci-Minafra I, Fontana S, Cancemi P, Basiricò L, Caricato S, et al. (2002) A contribution to breast cancer cell proteomics: detection of new sequences. Proteomics 2: 919-927.
13. Musso R, Di Cara G, Albanese NN, Marabeti MR, Cancemi P, et al. (2013) Differential proteomic and phenotypic behaviour of papillary and anaplastic thyroid cell lines. J Proteomics 90: 115-125.
14. DI Cara G, Marengo G, Albanese NN, Marabeti MR, Musso R, et al. (2013) Proteomic profiling of Trastuzumab (Herceptin(R))-sensitive and -resistant SKBR-3 breast cancer cells. Anticancer Res 33: 489-503.
15. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4: 44-57.
16. Pucci-Minafra I (2014) Breast Cancer Proteomics. In: Bahr D (2014) Omics Approaches in Breast Cancer. Springer India, New Delhi.
17. Cancemi P, Di Cara G, Albanese NN, Costantini F, Marabeti MR, et al. (2010) Large-scale proteomic identification of S100 proteins in breast cancer tissues. BMC Cancer 10: 476.
18. Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, et al. (2013) Functions of S100 proteins. Curr Mol Med 13: 24-57.
19. Bresnick AR, Weber DJ, Zimmer DB (2015) S100 proteins in cancer. Nat Rev Cancer 15: 96-109.
20. Chen H, Xu C, Jin Q, Liu Z (2014) S100 protein family in human cancer. Am J Cancer Res 4: 89-115.
21. Naz S, Bashir M, Ranganathan P, Bodapati P, Santosh V, et al. (2014) Protumorigenic actions of S100A2 involve regulation of PI3/Akt signaling and functional interaction with Smad3. Carcinogenesis 35: 14-23.
22. Gross SR, Sin CG, Barraclough R, Rudland PS (2014) Joining S100 proteins and migration: for better or for worse, in sickness and in health. Cell Mol Life Sci 71: 1551-1579.
23. Maletzki C, Bodammer P, Breitrück A, Kerkhoff C (2012) S100 proteins as diagnostic and prognostic markers in colorectal and hepatocellular carcinoma. Hepat Mon 12: e7240.
24. Mueller A, Schäfer BW, Ferrari S, Weibel M, Makek M, et al. (2005) The calcium-binding protein S100A2 interacts with p53 and modulates its transcriptional activity. J Biol Chem 280: 29186-29193.
25. Bulk E, Sargin B, Krug U, Hascher A, Jun Y, et al. (2009) S100A2 induces metastasis in non-small cell lung cancer. Clin Cancer Res 15: 22-29.
26. Nedjadi T, Kitteringham N, Campbell F, Jenkins RE, Park BK, et al. (2009) S100A6 binds to annexin 2 in pancreatic cancer cells and promotes pancreatic cancer cell motility. Br J Cancer 101: 1145-1154.
27. Cancemi P, Di Cara G, Albanese NN, Costantini F, Marabeti MR, et al. (2012) Differential occurrence of S100A7 in breast cancer tissues: a proteomic-based investigation. Proteomics Clin Appl 6: 364-373.
28. Ye L, Sun PH, Martin TA, Sanders AJ, Mason MD, et al. (2013) Psoriasin (S100A7) is a positive regulator of survival and invasion of prostate cancer cells. Urol Oncol 31: 1576-1583.
29. Zhou G, Xie TX, Zhao M, Jasser SA, Younes MN, et al. (2008) Reciprocal negative regulation between S100A7 / psoriasin and beta-catenin signaling plays an important role in tumor progression of squamous cell carcinoma of oral cavity. Oncogene 27: 3527-3538.
30. Markowitz J, Carson WE 3rd (2013) Review of S100A9 biology and its role in cancer. Biochim Biophys Acta 1835: 100-109.
31. Kwon CH, Moon HJ, Park HJ, Choi JH, Park do Y (2013) S100A8 and S100A9 promotes invasion and migration through p38 mitogen-activated protein kinase-dependent NF-κB activation in gastric cancer cells. Mol Cells 35: 226-234.
32. Xiao MB, Jiang F, Ni WK, Chen BY, Lu CH, et al. (2012) High expression of S100A11 in pancreatic adenocarcinoma is an unfavorable prognostic marker. Med Oncol 29: 1886-1891.
33. Pierce A, Barron N, Linehan R, Ryan E, O'Driscoll L, et al. (2008) Identification of a novel, functional role for S100A13 in invasive lung cancer cell lines. Eur J Cancer 44: 151-159.
34. Chen H, Yuan Y, Zhang C, Luo A, Ding F, et al. (2012) Involvement of S100A14 protein in cell invasion by affecting expression and function of matrix metalloproteinase (MMP)-2 via p53-dependent transcriptional regulation. J Biol Chem 287: 17109-17119.
35. Muller S, Berger M, Lehembre F, Seeler JS, Haupt Y, et al. (2000) c-Jun and p53 activity is modulated by SUMO-1 modification. J Biol Chem 275: 13321-13329.
36. Fulda S, Gorman AM, Hori O, Samali A (2010) Cellular stress responses: cell survival and cell death. Int J Cell Biol 2010: 214074.
37. Taipale M, Tucker G, Peng J, Krykbaeva I, Lin ZY, et al. (2014) A quantitative chaperone interaction network reveals the architecture of cellular protein homeostasis pathways. Cell 158: 434-448.
38. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. Trends Biochem Sci 31: 164-172.
39. Jia H, Halilou AI, Hu L, Cai W, Liu J, et al. (2011) Heat shock protein 10 (Hsp10) in immune-related diseases: one coin, two sides. Int J Biochem Mol Biol 2: 47-57.
40. Kim LS, Kim JH (2011) Heat shock protein as molecular targets for breast cancer therapeutics. J Breast Cancer 14: 167-174.
41. Zheng J, Lu Z, Kocabas F, BÃ¶ttcher RT, Costell M, et al. (2014) Profilin 1 is essential for retention and metabolism of mouse hematopoietic stem cells in bone marrow. Blood 123: 992-1001.
42. Chua BT, Volbracht C, Tan KO, Li R, Yu VC, et al. (2003) Mitochondrial translocation of cofilin is an early step in apoptosis induction. Nat Cell Biol 5: 1083-1089.
43. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, et al. (2011) Role of YAP/TAZ in mechanotransduction. Nature 474: 179-183.
44. Wang W, Eddy R, Condeelis J (2007) The cofilin pathway in breast cancer invasion and metastasis. Nat Rev Cancer 7: 429-440.
45. Tang MC, Chan LC, Yeh YC, Chen CY, Chou TY, et al. (2011) Thymosin beta

4 induces colon cancer cell migration and clinical metastasis via enhancing ILK / IQGAP1 / Rac1 signal transduction pathway. Cancer Lett 308: 162-171.

46. Pakozdi A, Amin MA, Haas CS, Martinez RJ, Haines GK, et al. (2006) Macrophage migration inhibitory factor: a mediator of matrix metalloproteinase-2 production in rheumatoid arthritis. Arthritis Res Ther 8: R132.

47. Simpson KD, Templeton DJ, Cross JV (2012) Macrophage migration inhibitory factor promotes tumor growth and metastasis by inducing myeloid-derived suppressor cells in the tumor microenvironment. J Immunol 189: 5533-5540.

48. Schimmack S, Taylor A, Lawrence B, Schmitz-Winnenthal H, Fischer L, et al. (2015) Stathmin in pancreatic neuroendocrine neoplasms: a marker of proliferation and PI3K signaling. Tumour Biol 36: 399-408.

49. Kimura K, Ojima H, Kubota D, Sakumoto M, Nakamura Y, et al. (2013) Proteomic identification of the macrophage-capping protein as a protein contributing to the malignant features of hepatocellular carcinoma. J Proteomics 78: 362-373.

50. Qu Y, Wang J, Ray PS, Guo H, Huang J, et al. (2011) Thioredoxin-like 2 regulates human cancer cell growth and metastasis via redox homeostasis and NF-ÎºB signaling. J Clin Invest 121: 212-225.

51. Zhang H, Kuai X, Ji Z, Li Z, Shi R (2013) Over-expression of small ubiquitin-related modifier-1 and sumoylated p53 in colon cancer. Cell Biochem Biophys 67: 1081-1087.

52. Wang Q, Xia N, Li T, Xu Y, Zou Y, et al. (2013) SUMO-specific protease 1 promotes prostate cancer progression and metastasis. Oncogene 32: 2493-2498.

53. Benanti JA, Williams DK, Robinson KL, Ozer HL, Galloway DA (2002) Induction of extracellular matrix-remodeling genes by the senescence-associated protein APA-1. Mol Cell Biol 22: 7385-7397.

54. Eck SM, Hoopes PJ, Petrella BL, Coon CI, Brinckerhoff CE (2009) Matrix metalloproteinase-1 promotes breast cancer angiogenesis and osteolysis in a novel in vivo model. Breast Cancer Res Treat 116: 79-90.

55. Nutter F, Holen I, Brown HK, Cross SS, Evans CA, et al. (2014) Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line. Endocr Relat Cancer 21: 327-341.