Abstract. Background/Aim: We examined the gene expression changes of breast cancer cells spontaneously undergoing epithelial–mesenchymal transition (EMT) and its reverse process mesenchymal–epithelial transition (MET) and the role of exosomes in these transitions. Materials and Methods: Highly invasive mesenchymal-like breast cancer cells, MDA-MB-231 (basal cells), EMT and MET variants, were characterized by microarray gene expression profiling, immunocytochemistry and chemosensitivity. Results: Spontaneously disseminated cells were anoikis resistant, exhibited a dissociative, EMT-like phenotype and underwent MET when reseeded in cell-free plates. MET was inhibited by exosomes secreted by basal cells. Chemo-sensitivity to doxorubicin, vincristine and paclitaxel decreased in the order EMT< MET< basal. Phenotypic plasticity arose with differential expression of metastasis and stemness associated genes (LGR5, FZD10, DTX1, ErbB3, FTH1 and DLL4) and pathways (DNA replication and repair, ABC transporter, Hedgehog, Notch and metabolic pathways). Conclusion: This is an appropriate model for studying EMT/MET transitions, drug targets and the role of exosomes in breast cancer dissemination.

Epithelial to mesenchymal transition (EMT) occurs during tumor progression and contributes to cellular plasticity endowing cancer cells with increased motility and invasiveness (1, 2). A critical molecular feature of EMT is the down-regulation of the adhesion molecule E-cadherin and the acquisition of a mesenchymal phenotype associated with the up-regulation of vimentin (3). When cancer cells successfully establish metastasis at secondary sites, they also undergo the reverse process known as mesenchymal–to–epithelial transition (MET) and re-acquire epithelial markers (4).

There are a number of experimental approaches able to reproduce EMT in vitro such as the forced expression of EMT-inducing transcription factors (5), transforming growth factor-β (TGFβ) treatment (6) or transfection with C35 (7). Trypsin-sensitive breast and colon cancer cells subpopulations also showed characteristic of EMT (8). Similarly, EMT-like phenotype can be increased by culturing cells on soft substrates or on ultra-low attachment plates (9, 10). These experimental manipulations artificially induced gene expression alterations and cellular phenotypes, which may not recapitulate the in vivo status of cells (11).

We have previously demonstrated that a subpopulation of adherent colon cancer cells spontaneously undergoes EMT and that this transition is stabilized by exosomes, since EMT cells, in an exosomes depleted environment, undergo the reverse process, MET (12). These findings demonstrate the possibility to study the inter-conversion between epithelial and mesenchymal states in vitro without any artificial experimental manipulation and in the absence of exogenous cues from the surrounding microenvironment. However, since the degree of the ability to undergo EMT may differ among tumors (13), in this study, we investigated whether EMT and MET variants and their associated traits, such as anoikis and therapy resistance, spontaneously emerge in the highly invasive mesenchymal-like breast cancer cells MDA-MB-231 and whether exosomes may be drivers of these transitions. Moreover, although previous reports described gene expression variation in experimentally induced EMT in vitro (14-16), to our knowledge, none sought to identify the dynamic gene expression changes leading to spontaneous acquisition of EMT/MET phenotypes.
**Materials and Methods**

**Cell culture and suspension cells.** MDA-MB-231 cells were maintained in DMEM (Invitrogen, Life technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin-streptomycin, 1% L-glutamine (200 mmol/l), 4.5 g/l glucose and grown in 5% CO2. These cells are referred as basal cells. After 7 days post seeding, a number of MDA-MB-231 cells able to survive in suspension appeared in the culture medium; these cells that spontaneously found in suspension (referred as EMT cells) were harvested by 500g centrifugation for 5 min and counted at different time points post seeding (48 h, 72 h, 96 h, 7 d, 10 d, 15 d).

**Exosomes isolation.** Exosomes were isolated from MDA-MB-231 culture media at day 7 post-seeding by using the Exosome Precipitation Solution (Macherey-Nagel, Düren, Germany).

**Adherent colonies formation.** To test the ability of EMT cells to form adherent colonies (referred as MET cells), 4x10^3 EMT cells collected at day 7 were seeded in 24-well plates and maintained for 72 h in DMEM or in DMEM containing 10% or 20% of exosomes isolated from basal cells culture media. MET cells were counted and cell viability was measured by trypan blue (Invitrogen).

**Immunocytochemistry for E-cadherin and vimentin.** Basal cells, as well as EMT and MET variants, were seeded on histological slides, fixed in 4% formaldehyde pH 7.4 for 10 min and pre-incubated in 0.5% triton and 1.5% bovine serum albumin (BSA) (Sigma Aldrich, Milan, Italy) for 15 min at RT. Immunostaining was performed by incubation for 24 h at 4°C with mouse monoclonal anti E-cadherin antibody at final dilution of 1:50 (Millipore, Burlington, MA, USA) or goat-polyclonal anti-Vimentin antibody at final dilution 1:40 (Sigma Aldrich) followed by Alexa Fluor 488 Goat anti-mouse (1:333) or Alexa Fluor 568 Donkey anti-Goat (1:333) antibodies (Invitrogen). Microscopic analysis was performed with a fluorescence microscope (Labophot-2, Nikon) connected to a CCD camera. Ten photomicrographs (~100 cells/microscopic field) were randomly taken for each sample and fluorescence was measured using ImageJ 1.33 image analysis software (http://rsb.info.nih.gov/ij). Results were expressed in arbitrary units.

**Chemosensitivity assay.** Differences in sensitivity among basal, EMT and MET cells exposed to doxorubicin (10^{-6} M), paclitaxel (10^{-6} M) or vincristine (10^{-6} M) for 72 h were assessed by MTS assay (Promega Corporation, Madison, WI, USA).

**Reverse Transcription PCR.** Total RNA was extracted by using the RNeasy Mini kit Plus (Macherey-Nagel) according to the manufacturer’s protocol. For first-strand cDNA synthesis, 1 μg of total RNA from each sample was reverse-transcribed by using the RevertAid RT Kit (Thermo Fisher Scientific, Waltham, MA, USA). Primers were designed based on the human GenBank sequences. For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to GAPDH mRNA (17).

**Microarray experiments.** To produce Cy3-labeled complementary RNA (cRNA), 550 ng of total RNA from the three cell clones, were labeled using the Agilent Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer’s protocol. The Cy3-labeled samples were hybridized to Agilent Whole human genome 4X44K microarrays at 65°C for 18 h. Fluorescent signal intensities were detected using the Agilent Scan Control 7.0 Software on an Agilent DNA Microarray Scanner, at a resolution of 2 μm. Microarray analysis was performed on 3 independent biological replicates for each cell clone. Image analysis and initial quality control were performed using Agilent Feature Extraction Software v9.5. Values for control spots and spots that did not meet the quality criteria were flagged. Quality criteria included a minimal spot size, a median/mean ratio of at least 0.9 for each spot, non-saturated intensity for both channels, a signal well above background and a minimal signal intensity for at least one channel. Initial statistical analysis was performed using unpaired t-test considering Benjamini–Hochberg corrected p-value of 0.05. BRB-Array Tools 4.5.1 version was used to perform Statistical Analysis of Microarray (SAM), Gene set enrichment analysis (GSEA) and a two-way hierarchical clustering. Microarray data are stored into the Array Express database with accession number E-MTAB-6954.

**Statistical analysis.** Data were expressed as means±SEM of three independent experiments. Statistical analysis was performed by one-way analysis of variance, followed by the Student–Newman–Keuls multiple comparison post hoc test or by Mann–Whitney test. Calculations were done using a GraphPad Prism 4.0 (GraphPad software, San Diego, CA, USA).

**Results**

**Behavioral characterization of suspension EMT cells and effect of exosomes secreted by basal cells on the reverse transition from EMT to MET.** At 48 h post-seeding, a subpopulation of MDA-MB-231 basal cells detached the monolayer and spontaneously disseminated in the culture media. The number of cells growing in suspension significantly increased from the seventh to the fifteenth day post-seeding suggesting anoikis resistance and EMT like features (Figure 1A). When 4x10^3 suspension cells were collected and re-seeded in cell-free wells, they were able to adhere again and to form about 300 new adherent viable colonies suggesting the occurrence of MET.

When exosomes isolated from the culture medium of basal cells were added at a concentration of 10% or 20% to EMT cells, their ability to undergo MET in cell-free wells was significantly and dose dependently reduced compared to DMEM alone (Figure 1B).

**Expression of the EMT hallmarks vimentin and E-cadherin.** Adherent MDA-MB-231 cells (Basal), had high expression levels of E-cadherin and low expression of vimentin (Figure 2A and B). On the contrary, disseminated MDA-MB-231 (EMT) were E-cadherin low and vimentin high (Figure 2C and D). When the latter where re-seeded in cell-free wells, they reverted back to the epithelial state forming new adherent viable colonies (MET), that were E-cadherin high and vimentin low (Figure 2E and F); Figure 2G and H show
the results of the densitometric analysis of E-cadherin and vimentin, respectively, in basal, EMT and MET cells. The gene expression of vimentin and E-cadherin recapitulated the results of immunocytochemistry with EMT cells displaying up-regulation of vimentin and down-regulation of E-cadherin compared to both adherent and MET cells (Table I).

**Chemosensitivity assay.** In basal cells, the percentage of cell death was of 28 %, 38 % and 42% upon doxorubicin, paclitaxel and vincristine treatment, respectively. These percentages were dramatically reduced in EMT cells which were almost insensitive to all the three chemotherapeutic agents with percentages of cell death below 10 % (p<0.001). The chemosensitivity to doxorubicin was completely restored in MET cells whereas that to paclitaxel and vincristine was only partially reacquired compared to basal cells (Figure 3).

**Whole-gene expression analysis and Gene Set Enrichment Analysis (GSEA).** Transcriptomic analysis identified 223 differentially expressed genes between EMT cells and basal cells; among the genes that were up-regulated in EMT compared to basal cells are DTX1, ADH1A, BCAS1, FZD10 and ERBB3; beta 1 catenin (CTNNB1) was instead down-regulated. 219 genes were found differentially expressed by comparing EMT and MET cells: FTH1, was up-regulated in EMT compared to MET cells whereas DLL4 was down-regulated. PBOV, BMP7, RHOJ, ELA2B and ELA3B were all up-regulated in MET, and were among the 319 differentially expressed genes between MET and basal cells.

Unsupervised hierarchical clustering analysis was performed in order to evaluate the degree of changes in gene expression observed in EMT and MET cells compared to basal cells. The results are shown in the form of a tree whose branch lengths reflect the degree of similarities among the experimental groups; thus, similar expression profiles are closer to each other. This analysis distinguished the expression profiles of the three cell clones and in particular those of EMT cells compared to basal cells (Figure 4A).

Venn diagrams showed the degree of overlapping results in gene expression among different comparisons (Figure 4B). In particular, the expression of a single gene, the heat shock protein HSP90AA, emerged from all the three comparisons and was up-regulated in basal and EMT and was down-regulated in MET. Interestingly, 7 genes whose expression regulation emerged from the comparison between basal and EMT and between EMT and MET were found. LGR5, SLC30A2 and COBL were overexpressed in EMT cells compared to basal and MET. Instead, CYTB, ADH5, ARL13B and ATP5B were...
Figure 2. Continued
highly expressed in basal and MET compared to EMT. When comparing MET cells to EMT and basal cells, 26 genes were differentially expressed: *PBOV1* was up-regulated in EMT and MET cells compared to basal cells, *FTH1* and *SIRT3* were up-regulated in basal and EMT compared to MET cells, and *ELA3B* was up-regulated in MET compared to basal and EMT cells.

In order to evaluate the biological processes characterizing the three cell clones, a Gene Set Enrichment Analysis (GSEA) was performed. This analysis measures the cumulative effect of small but consistent changes in gene expression within a biological pathway. Forty-six out of 136 KEGG pathways passed the 0.001 significance threshold. Table II shows the first 22 gene sets of the list. Among them, genes associated to the DNA replication (hsa03030), base excision repair (hsa03410) and TCA cycle (hsa00020) were mainly up-regulated in MET compared to basal and EMT cells (Figure 5). Other gene sets significantly enriched in EMT and MET cells were mTOR, NOD-like receptor signaling and several metabolic pathways.

Moreover, comparing each cell variant to the other ones, the Hedgehog signaling pathway found to be down-regulated in basal cells, became up-regulated in EMT and returned to a down-regulated state in MET. ABC transporters were mostly up-regulated in MET cells, drug metabolism-associated genes were overall down-regulated in basal cells, whereas both EMT and MET showed an up-regulation of this gene set. Notch was up-regulated in EMT cells compared to both basal and MET cells (Figure 6).

GSEA identified also 48 gene sets, computationally predicted to be targets of the same miRNA, that were differentially expressed in EMT, MET and basal cells (Table III). As an example, Figure 7, shows the heat maps with the list of genes predicted to be targets of miR-30b, miR-181a, and miR134.

### Discussion

Metastasis is the major cause of treatment failure and death in breast cancer patients (18). For many years, EMT has been seen as an extreme phenotype, recapitulated *in vitro* with artificial manipulations which keep cells in a fixed state. However, there is evidence that, *in vivo*, cancer cells undergo a spectrum of intermediate states called metastable phenotypes in which cells retain some epithelial features but also acquire mesenchymal characteristics (19). These hybrid phenotypes that seem to be facilitated in collective cell migration, are associated with the acquisition of stem-like properties, chemoresistance and aggressiveness (20-21).
These observations illustrate the importance of studying EMT in a non-experimentally manipulated setting (11). Moreover, most of the in vitro studies conducted so far, focused on EMT transition rather to its reverse process MET. Park et al. recently demonstrated that a long-term suspension of MDA-MB-468 cells resembled some features of human circulating tumor cells (10), but did not investigate their ability to overcome the EMT program, which is essential for metastatic colonization.

In line with our previous results on HCT8 colon cancer cells, we demonstrated that EMT transition spontaneously occurs also in MDA-MB-231 cells, and that exosomes
MET cells are molecularly distinct variants compared to parental cells. However, our results suggest that at least part of the plasticity cultured with hepatocytes, suggesting that micro the elegant paper from Chao and coworkers, MDA-MB-231 exhibited a reversion to an epithelial phenotype when completely re-acquired and that of vincristine and paclitaxel, the most commonly used drugs for the therapy of breast cancer. Interestingly, upon the acquisition of the MET phenotype, the sensitivity to doxorubicin is completely re-acquired and that of vincristine and paclitaxel is significantly regained, but still reduced compared to parental cells.

Whole gene expression analysis showed that EMT and MET cells are molecularly distinct variants compared to adherent cells. Given the large number of genes differentially expressed, attention was paid on those expressed in an opposite manner almost in two out of the three cell clones, as it was hypothesized that metastasis-related genes are more likely to be oppositely expressed. A number of genes associated with metastatic activity and cancer stem cell traits were identified. Among those are the leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5), a stem cell marker in intestinal crypts and mammary glands (23) that promotes cell mobility, tumor formation and EMT in breast cancer cells by activating Wnt/β-catenin signaling (24), FZD10, a receptor for Wnt signalling, that is up-regulated in breast cancer and is a potential drug target (25), DTX1, a Notch interacting protein that is also associated with the proliferative, migratory and clonogenic potential of cancer cells (26) and elevated expression of ErbB3 resulting in paclitaxel resistance in breast cancer cells (27).

Among the most up-regulated genes in EMT cells compared to MET cells, was the FTTH1 gene, a subunit of the ferritin complex, which is associated with the progression of breast cancer and with increased resistance to doxorubicin (28). DLL4 (Delta-like 4), a component of the Notch signaling pathway, has also been implicated in EMT and chemoresistance (29).
The unique gene significantly modulated in the three cell variants was HSP90AA1, a member of the HSP90 family whose expression is an independent risk factor of death from metastatic breast cancer in TNBC (30) and it is associated to chemoresistance (31). Pharmacological inhibitors of HSP90 in breast cancer treatment are under investigation in clinical trials (32).

An alternative approach to the analysis of single gene expression variations, is GSEA analysis, where a group of related genes from the same pathway is examined instead of groups of potentially unrelated genes. This approach has the advantage of taking into account the cooperative nature of genes and of considering that genes involved in the same process are often regulated together. Among the gene sets found to be differentially expressed among the three cell variants the DNA replication end Base Excision Repair was found; most of the genes listed in these pathways were in fact up-regulated in MET cells, suggesting that both mechanisms might be involved in cell survival and chemoresistance after re-adhesion. TCA cycle, mTOR, NOD-like receptor signaling and several metabolic gene sets were also enriched in EMT and MET cells. Hedgehog signaling pathways were clearly down-regulated in basal cells, became up-regulated in EMT cells and returned to a down-regulated state in MET cells. A similar trend was observed for Notch pathway; interestingly, both contribute to the maintenance of stem-like properties and favor chemoresistance and metastasis (33). ABC transporters and drug metabolism pathways were activated in MET cells and in both EMT and MET cells respectively, suggesting their potential role in the acquisition of chemoresistance. GSEA also identified several gene sets predicted to be targets of the same miRNA; the most differentially expressed gene set was that associate with the miR-30d recently recognized as a mediator of invasion, migration and EMT in breast cancer (34).

Figure 5. Heat maps representing the three KEGG pathways pointed out by Gene Set Enrichment Analysis (GSEA) of MDA-MB-231 cells transcriptomic data: DNA replication (hsa03030), base excision repair (hsa03410) and TCA cycle (hsa00020). Up-regulated genes are shown in dark blue, down-regulated genes in light blue color.
In conclusion, evidence was provided indicating that a dynamic gene expression reprogramming during EMT-MET takes place in vitro without any experimental manipulation and that it is associated to the acquisition of chemoresistance and metastatic traits. This model is useful for studying EMT and MET transitions, drug targets and the role of tumor-derived exosomes in breast cancer.

Conflicts of Interest

The Authors report no conflict of interest regarding this study.

Authors’ Contributions

EB and LC conceived and designed the project. CL designed and performed microarray experiments and supervised the project; LC and MD performed cell cultures and carried out immunocytochemistry; EB and CL performed microarray analysis and interpretation; EB and CL wrote the manuscript. All authors read and approved the final version of the manuscript.

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Figure 7. Heat maps representing the gene sets predicted to be targets of miR-30d, miR-181a and miR-134. Up-regulated genes are shown in dark blue, down-regulated genes in light blue color.
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