

# Transcriptomic Characterization, Chemosensitivity and Regulatory Effects of Exosomes in Spontaneous EMT/MET Transitions of Breast Cancer Cells

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**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 chemo-sensitivity. 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- $\beta$  (TGF $\beta$ ) 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.

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*Key Words:* Breast cancer, epithelial–mesenchymal transition (EMT), mesenchymal–epithelial transition (MET), metastasis, chemoresistance, exosomes.

## 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% CO<sub>2</sub>. 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 500xg 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), 4×10<sup>3</sup> 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<sup>-6</sup> M), paclitaxel (10<sup>-6</sup> M) or vincristine (10<sup>-6</sup> 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 4×10<sup>3</sup> 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 were 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

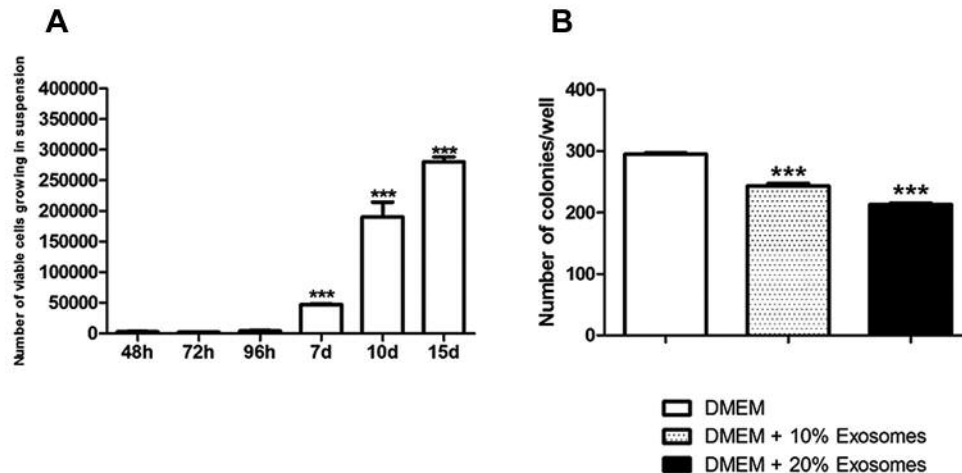


Figure 1. Behavioral characterization of suspension EMT cells and effect of exosomes secreted by basal cells on the reverse transition from EMT to MET. A: Time-dependent increase in the number of viable MDA-MB-231 cells spontaneously growing in suspension. Data are expressed as means $\pm$ SEM of 3 independent experiments. \*\*\* $p$ <0.001 vs. 48 h. B: Number of adherent new colonies (MET cells) formed by EMT cells in DMEM alone or in the presence of 10% and 20% exosomes isolated from the culture medium of basal cells. \*\*\* $p$ <0.001 vs. DMEM alone.

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.

Table I. Expression of vimentin and E-cadherin, in basal, EMT and MET.

Gene	Basal	EMT	MET
Vimentin	0.39 $\pm$ 0.03	0.78 $\pm$ 0.05**	0.31 $\pm$ 0.02##
E-Cadherin	0.43 $\pm$ 0.04	ND**	0.22 $\pm$ 0.01#

Data are expressed as mean $\pm$ SEM of the relative amount of each gene vs. the housekeeping gene, GAPDH. \* $p$ <0.05, \*\* $p$ <0.01 vs. Basal; # $p$ <0.05, ## $p$ <0.01 vs. EMT.

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 cells compared to 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

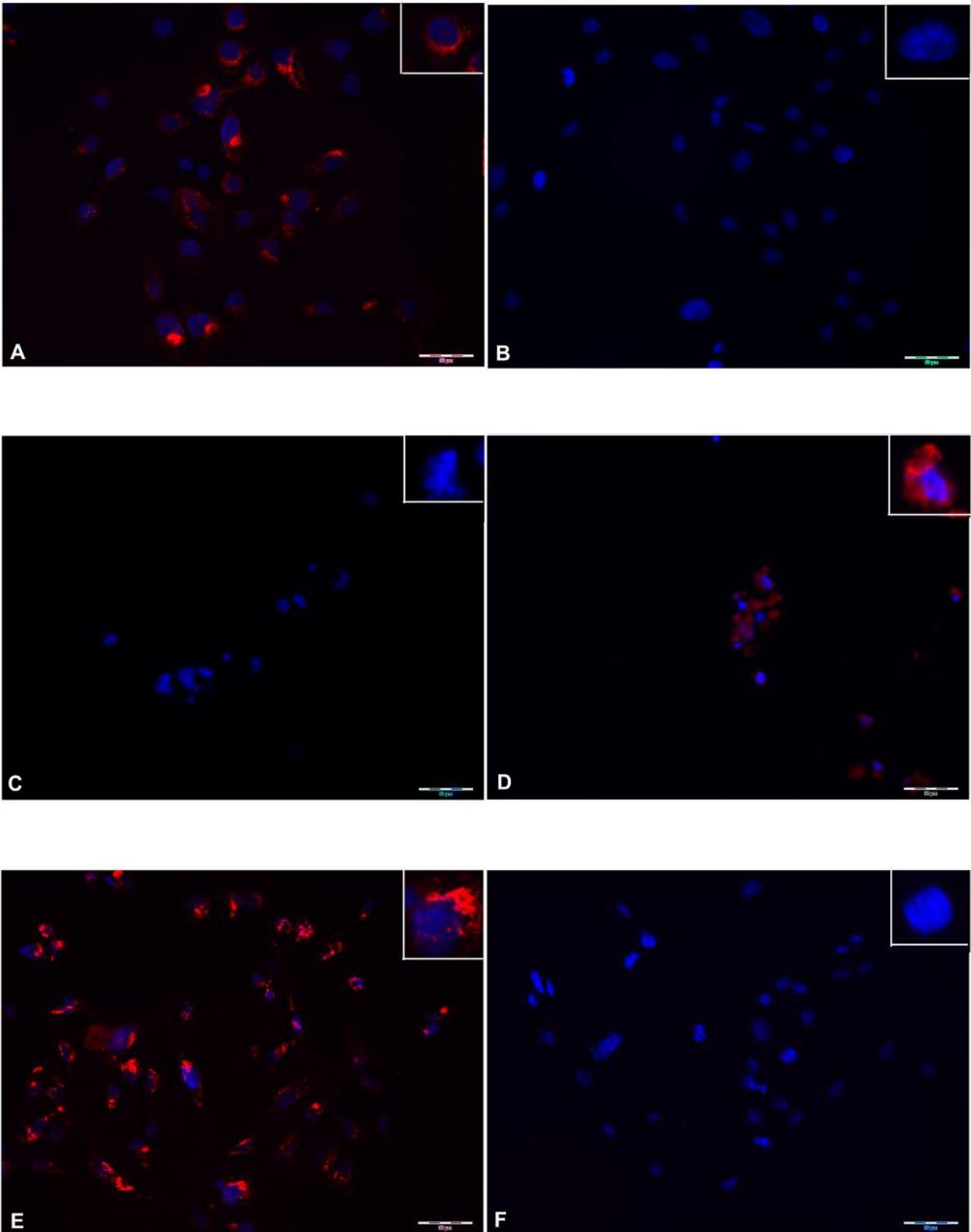


Figure 2. *Continued*

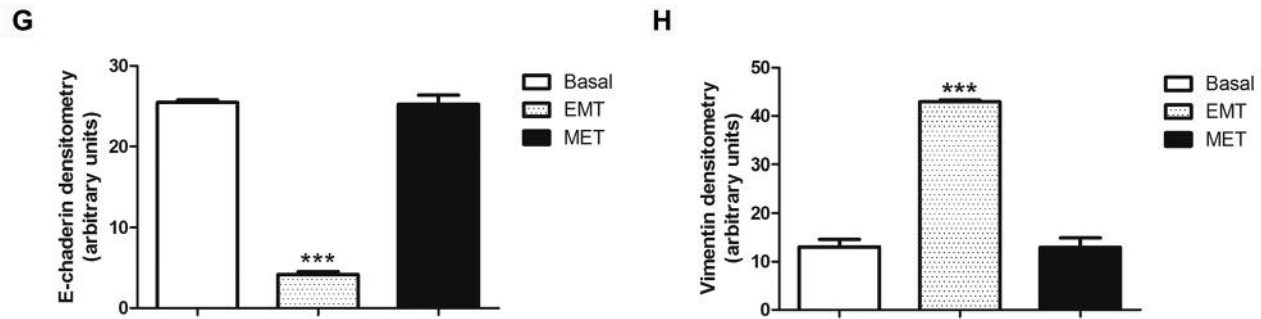


Figure 2. Expression of the EMT hallmarks vimentin and E-cadherin. Immunocytochemical evaluation of the expression of E-cadherin (A) and vimentin (B) in adherently growing MDA-MB-231 cells (Basal). Expression of E-cadherin (C) and vimentin (D) in MDA-MB-231 growing in suspension (EMT). Expression of E-cadherin (E) and vimentin (F) in adherent new colonies (MET) obtained by seeding disseminated MDA-MB-231 cells in cell-free wells (20x magnification; Barr 100 mm objective). Densitometric analysis of E-cadherin in Basal, EMT and MET cells (G). Densitometric analysis of vimentin in Basal, EMT and MET cells.  $^{***}p < 0.01$  vs. Basal and MET cells (H).

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.

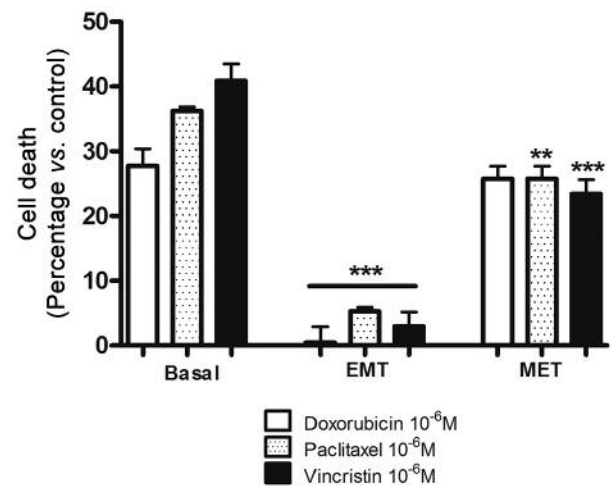


Figure 3. Chemosensitivity assay in basal, EMT and MET cells. Data are expressed as means  $\pm$  SEM of 3 independent experiments.  $^{***}p < 0.001$  vs. adherent MDA-MB-231.

## 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).

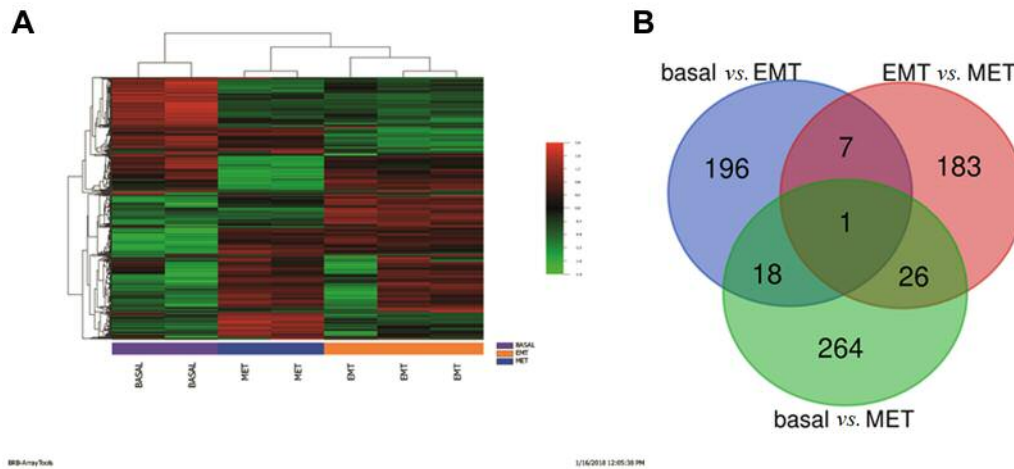


Figure 4. Gene Expression Analysis. A: Hierarchical clustering of gene expression profiles of EMT, MET and basal cells. B: Venn diagram showing the number of differentially expressed genes in the three cell clones, the degree of overlap between the differentially expressed genes in the three comparisons; non-overlapping numbers specify the genes unique to each comparison.

Table II. Top 22 KEGG gene sets found to be differentially modulated by comparing basal, EMT and MET cells.

Kegg Pathway	Pathway description	Number of genes	LS permutation p-Value	KS permutation p-Value
hsa05219	Bladder cancer	65	0.00001	0.00005
hsa05221	Acute myeloid leukemia	96	0.00003	0.00001
hsa04150	mTOR signaling pathway	83	0.00005	0.00014
hsa05120	Epithelial cell signaling in <i>Helicobacter pylori</i> infection	98	0.00008	0.00001
hsa05223	Non-small cell lung cancer	82	0.00008	0.00015
hsa03030	DNA replication	47	0.00009	0.00001
hsa04621	NOD-like receptor signaling pathway	79	0.00011	0.00011
hsa05213	Endometrial cancer	87	0.00017	0.0018
hsa00310	Lysine degradation	78	0.00032	0.00136
hsa05130	Pathogenic <i>Escherichia coli</i> infection	91	0.00077	0.00002
hsa03008	Ribosome biogenesis in eukaryotes	98	0.00083	0.00001
hsa00450	Seleno compound metabolism	22	0.00089	0.00094
hsa03410	Base excision repair	54	0.00159	0.00001
hsa05214	Glioma	100	0.00168	0.00082
hsa00020	Citrate cycle (TCA cycle)	48	0.00209	0.00047
hsa03022	Basal transcription factors	49	0.00224	0.00001
hsa04962	Vasopressin-regulated water reabsorption	78	0.0025	0.00024
hsa00330	Arginine and proline metabolism	76	0.00638	0.00035
hsa00520	Amino sugar and nucleotide sugar metabolism	65	0.00704	0.00097
hsa00630	Glyoxylate and dicarboxylate metabolism	27	0.01191	0.00069
hsa04146	Peroxisome	98	0.01792	0.00002
hsa00280	Valine, leucine and isoleucine degradation	68	0.02396	0.00039

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

Table III. Top 22 gene sets computationally predicted to be targets of the same miRNA, found to be differentially modulated by comparing basal, EMT and MET cells.

	MicroRNA GeneSets	Number of genes	LS permutation $p$ -Value	KS permutation $p$ -Value	Efron-Tibshirani's GSA test $p$ -Value
1	hsa-miR-30d-5p	12	0.00001	0.00009	0.055
2	hsa-miR-424-5p	37	0.00001	0.00254	0.285
3	hsa-miR-133a-3p	39	0.00001	0.00063	0.285
4	hsa-miR-503-5p	23	0.00004	0.00284	<0.005
5	hsa-miR-24-3p	61	0.00006	0.00048	0.345
6	hsa-miR-221-3p	52	0.00007	0.00005	0.195
7	hsa-miR-181a-5p	44	0.00009	0.00003	0.215
8	hsa-miR-26a-5p	56	0.00009	0.00015	0.255
9	hsa-miR-134-5p	6	0.00011	0.00498	0.105
10	hsa-miR-199a-5p	23	0.00013	0.0021	0.055
11	hsa-miR-143-3p	27	0.00015	0.00001	0.3
12	hsa-miR-183-5p	13	0.00015	0.00306	0.11
13	hsa-miR-141-3p	63	0.0002	0.00177	0.605
14	hsa-miR-15a-5p	36	0.00028	0.04089	0.275
15	hsa-miR-222-3p	35	0.00033	0.00154	0.19
16	hsa-miR-200a-3p	46	0.00058	0.01587	0.25
17	hsa-miR-661	9	0.00082	0.00084	0.335
18	hsa-miR-25-3p	18	0.00085	0.00384	0.195
19	hsa-miR-193b-3p	21	0.00089	0.01751	0.29
20	hsa-miR-16-5p	40	0.0009	0.02266	0.545
21	hsa-let-7c-5p	16	0.00092	0.00048	0.025
22	hsa-miR-127-3p	8	0.001	0.00298	0.18

stabilize the EMT phenotype allowing anoikis resistance; when exosomes are removed, EMT cells revert back to the epithelial state, undergoing MET. *In vivo*, it is likely that a complex mix of signals, both from cancer cells and from the tumor microenvironment, contribute to these transitions: in the elegant paper from Chao and coworkers, MDA-MB-231 exhibited a reversion to an epithelial phenotype when cultured with hepatocytes, suggesting that micro environmental factors may modulate this switch (22). However, our results suggest that at least part of the plasticity of the EMT and MET phenotypes can be driven by exosomes, reinforcing the idea that by secreting exosomes, primary cancer cells try to preserve their own niche meanwhile endowing cells with migratory and invasive traits necessary to colonize other free niches (12).

In addition, EMT cells are selectively advantaged through the acquisition of chemoresistance to doxorubicin, 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/ $\beta$ -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 *FTH1* 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).



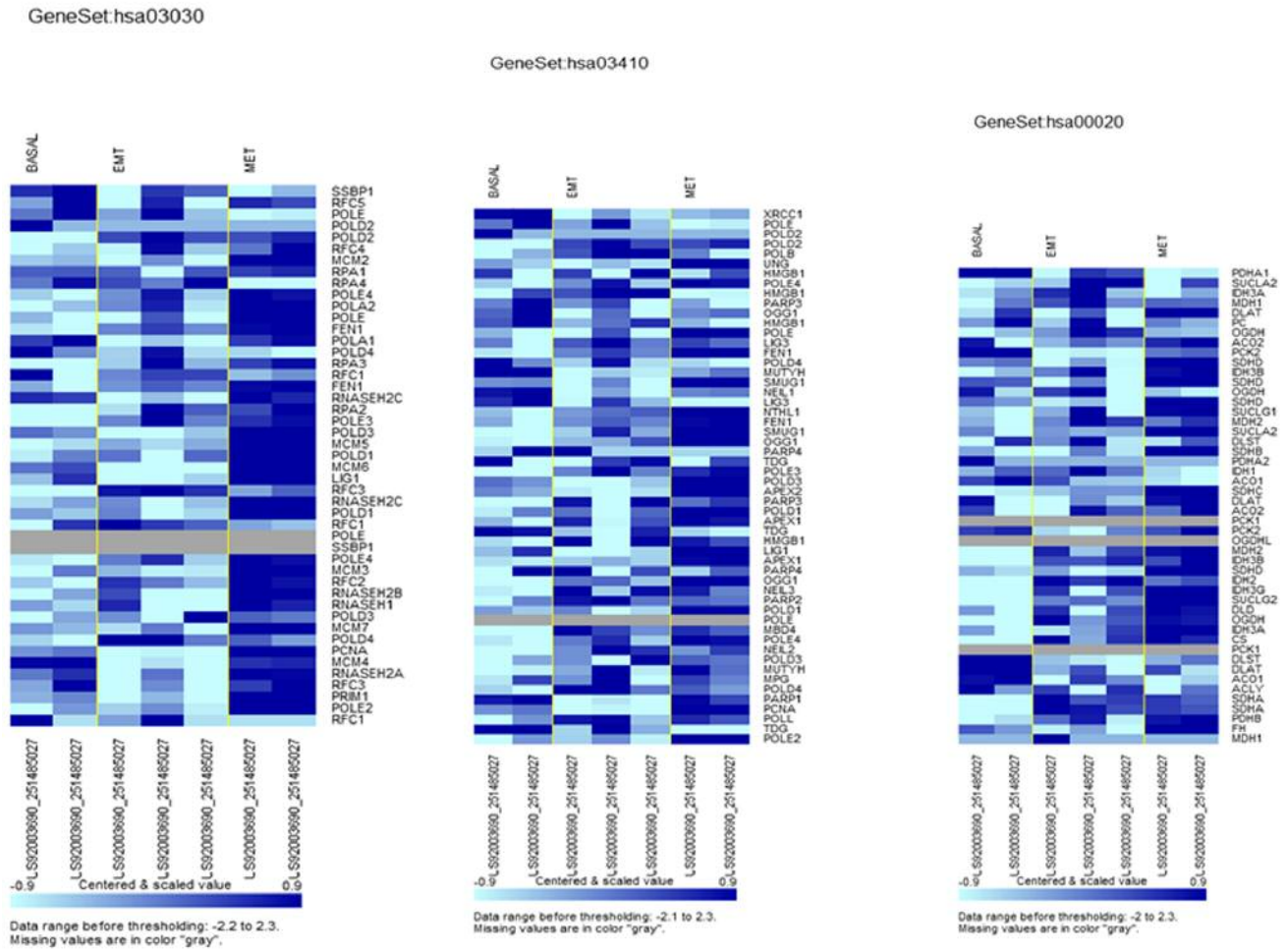


Figure 5. Heat maps representing the three KEGG pathways pointed out by Gene Set Enrichment Analysis (GSEA) of MDA-MB-231 cells transcriptomic data: DNA replication (hsa03030), base excision repair (hsa03410) and TCA cycle (hsa00020). Up-regulated genes are shown in dark blue, down-regulated genes in light blue color.

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).



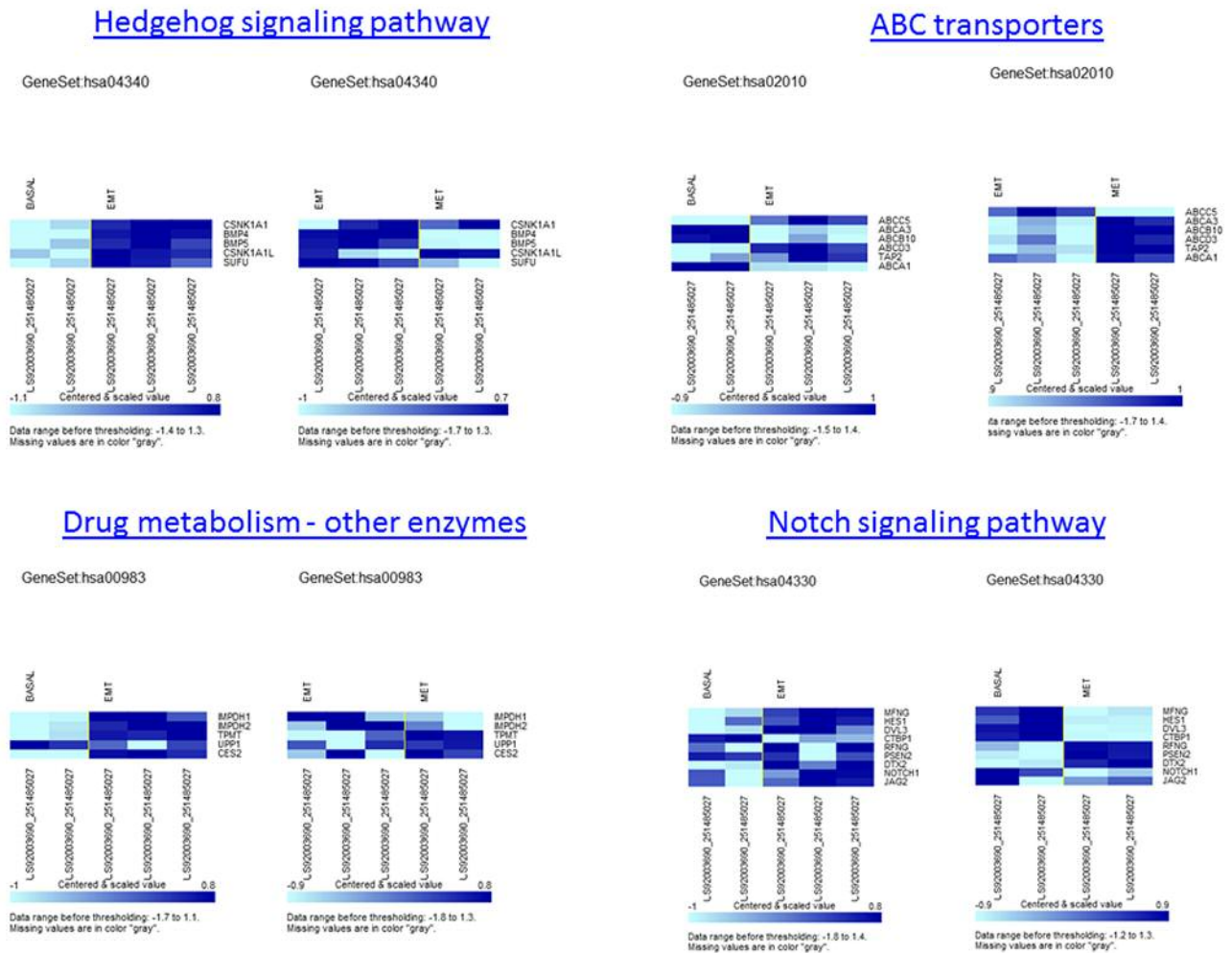


Figure 6. Heat maps representing KEGG pathways pointed out by Gene Set Enrichment Analysis (GSEA) of MDA-MB-231 cells transcriptomic data. Up-regulated genes are shown in dark blue, down-regulated genes in light blue color.

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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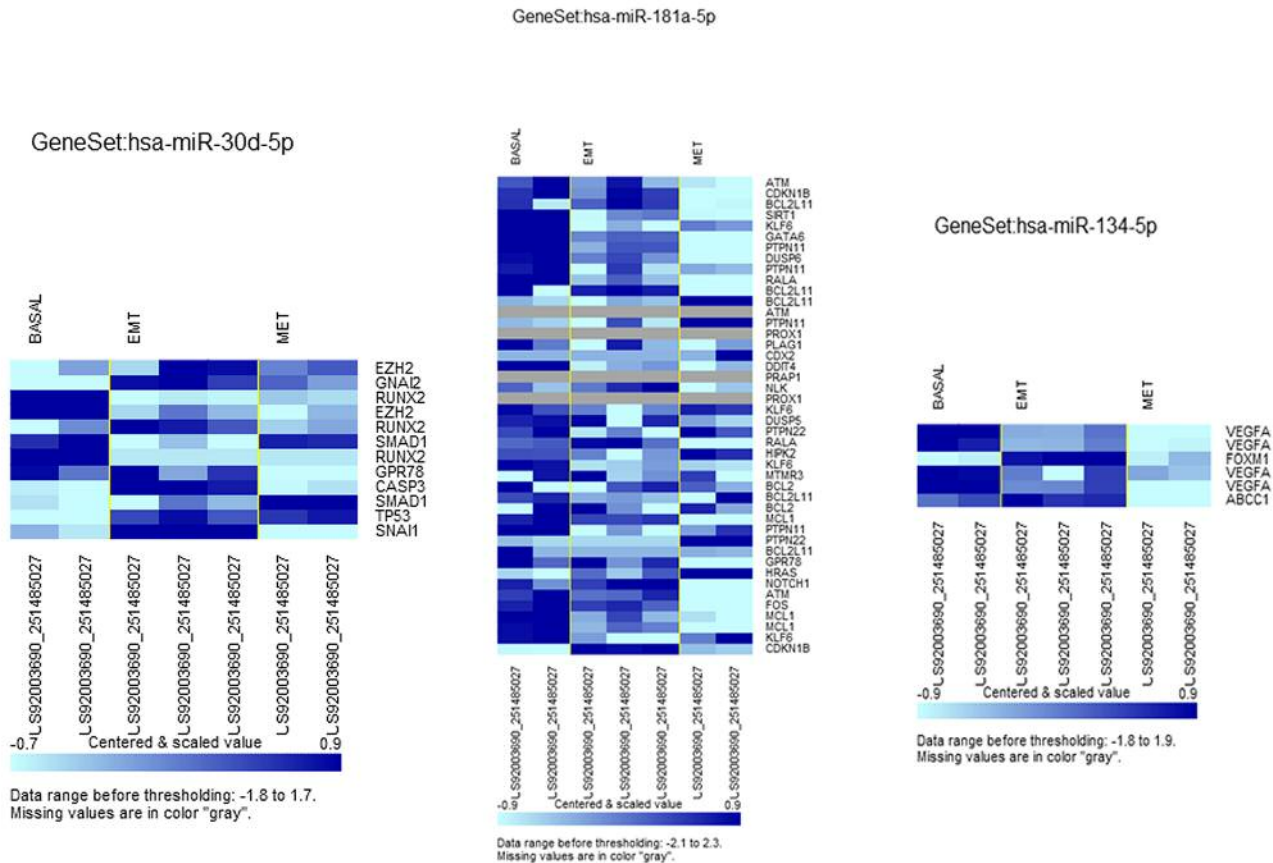


Figure 7. Heat maps representing the gene sets predicted to be targets of miR-30d, miR-181a and miR-134. Up-regulated genes are shown in dark blue, down-regulated genes in light blue color.

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