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The close relation between Epithelial-to-Mesenchymal Transition and Stem phenotype in Breast Cancer

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TABLE OF CONTENTS

ABSTRACT

In breast cancer, compelling evidence reveal that stemness and EMT (Epithelial-to-Mesenchymal transition) are strictly connected phenomena. Within human breast cancer, $CD44^{+}/CD24^{-/low}$ antigenic phenotype identifies cells with stem properties, which is markedly represented in the basal-like breast cancer subtype. These tumors are mostly triple negative (ESR1-/PGR-/HER2-), are associated with aggressive behavior and are also characterized by diffuse phenomena of EMT, that causes gain of mesenchymal markers and loss of epithelial/luminal markers. EMT is triggered by the activation of transcription factors, among which ZEB1, whose ectopic expression also correlates with the gain of stem cells properties.

The hypothesis underling this study is that common determinants of both EMT and stemness exist and may control the metastatic potential of breast tumors. Thus, the understanding of the mechanisms underpinning the EMT and stem phenotype may provide valuable tools for efficient therapies targeting the roots of metastasis.

With the aim of investigating the role of stem enriching culture conditions in modulating EMT and stem phenotypes, we characterized a panel of ten cell lines including luminal and triple negative breast cancer cell models in standard and non-differentiating cultures conditions in term of mesenchymal, epithelial and stem signatures.

Then, we evaluated the effect of silencing of known EMT-driver genes (particularly ZEB1) on EMT and stem features. The loss of function experiments demonstrated a cooperation of ZEB1 and aberrant methylation in the control of miR200c and E-cadherin expression. Furthermore, depletion of ZEB1 caused a shrinkage of stem population that seemed to be independent from the induction of miR200c. Importantly, we observed that ZEB1 might affect the response to EGFR-inhibitors.

This provided information on the markers mainly modulated in the EMT and stem reversion to use in a high-throughput screen based on shRNA-library technology aimed at identifying novel molecular determinants of EMT and CSC. In this screen, we considered to recognize genes whose silencing resulted in reversion of EMT and loss of stem properties in breast cancer model. Thus, E-cadherin and CD24 or EpCAM might be used as a readouts in the sorting of EM- and stem-reverted cells, respectively. Given the complexity of this reverse genetic screen, within the time frame of the PhD, we accomplished the setting-up phase.

INTRODUCTION

A breast cancer overview

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer mortality in females worldwide, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths in 2008 *(Jemal, 2011)*. Despite advances in diagnosis and treatment, breast cancer remains the second highest cause of death in developing countries.

In recent years, although many therapeutic strategies were developed to treat breast cancer, including surgery, chemo- and radiotherapy, and endocrine therapy, a large number of patients relapse after an initial response to treatments. In fact, the majority of breast cancer-related morbidity and mortality is principally attributable to the development and dissemination of metastases from the primary tumor site to distant organs *(Sreekumar, 2011)*.

Breast cancer represents a complex and heterogeneous disease that comprise distinct histological features, therapeutic response, dissemination patterns to distant sites and patient outcomes. The heterogeneity of tumors observed in histology has been used for disease classification for a long time. Advent of high-throughput technologies, in particular transcriptional profiling based on microarrays analysis, provided additional insights to explain heterogeneity of breast cancer and to classify cancer patients. At least, four molecular subtypes of invasive breast cancer have been established: luminal A, luminal B, HER2-enriched and basal-like and a normal breast-like group Each of these subtypes reflects different risk factors for response to treatment, disease progression and preferential sites of metastases *(Perou, 2000; Sorlie, 2001)*. Luminal tumors are positive for estrogen and progesterone receptors (ER and PR, respectively) and luminal cytokeratins (CK) CK8, CK18 and CK19. Among them, luminal A tumors are typically low grade, weakly proliferative and invasive, thus associated with a favorable prognosis. In contrast, luminal B tumors express a higher level of proliferation-related genes and HER2 (human epidermal growth factor receptor 2) compared to luminal A. HER2-enriched tumors typically over express or amplify HER2 oncogene and express HER2-amplicon associated genes, although HER2 confer high malignant phenotype to these tumors, they can be effectively targeted with different combination of specific anti-HER2 therapies.

Finally, basal-like tumors are characterized by high expression levels of basal/myo-epithelial markers including cytokeratins (CK) CK5/6, CK14, CK17 and the epidermal growth factor receptor (EGFR); distinctively, they lack hormone receptors and HER2, hence referred to as triple negative breast cancers (ER-, PR- and HER2-). However, because molecular profile are not always available, the lack of expression of ER, PR and HER2 are routinely used as bona fide representative of basal-like breast cancer (triple-negative breast cancer) *(Prat, 2011; Perou, 2010)*.

In general, triple-negative tumors are a minority of breast cancer (10-20%) but represent an important clinical challenge because of their poor prognosis. In fact, these cancers are highly proliferative due to the high incidence of p53 mutations, scarcely respond to standard cure and lack a tailored therapy *(Carey, 2011)*.

Interestingly, more recently a potential new group close to the basal-like subtype was described by genome profiling and referred as "claudin-low" both in human and mouse tumors *(Herschkowitz, 2007)*. Clinically, the majority of Claudin-low tumors represent only 5% about of all breast tumors and are poor prognosis triple-negative invasive ductal carcinoma with a high frequency of opposite cell types not found in normal breast epithelium referred as metaplastic and medullary differentiation *(Prat, 2011)*. Claudin-low subtype was characterized by the low expression of genes involved in tight junctions and cell-cell adhesion, including claudin -3,-4,-7, occludin and E-cadherin. Importantly, these tumors are characterized by high mesenchymal features and low luminal epithelial differentiation. Albeit these tumors are closely related to the basal-like subtype and share features with them, they are particularly enriched with unique properties linked to normal and cancer stem cells (CSCs) *(Lim, 2009; Hennessy, 2009)* and display an EMT (epithelial-to-mesenchymal transition) gene signature *(Taube, 2010; Prat, 2010)*.

Genetic and transcriptional characteristics of breast tumors are well represented in breast cancer cell lines *(Neve, 2006; Blick, 2008; Hollestelle, 2009; Prat, 2010)*. Cell lines have been widely used to investigate breast cancer patho-biology and to screen and characterise new therapeutic agents.

Analysis of transcriptional expression pattern of breast cell lines revealed two major clusters: luminal and basal. The luminal cluster included ER+ and/or HER2+ cell lines (MCF7, T47D, BT474, SKBR3), while the basal subgroup is further divided into A and B subgroups (*Neve,*

2006). Basal-A group (MDA468) matched closely to the basal-like signature found in the primary tumors; Basal-B, instead, displays similar gene expression patterns of claudin-low human tumors, namely low expression of the luminal and HER2 gene clusters, scarce expression of the basal group, and low expression of the cell-cell adhesion cluster as well as mutations in p53 gene (HBL100, Hs578T, MDA-MB157, MDA-MB 231 and MDA-MB436). As for claudin-low tumors, accumulating evidence suggest that these claudin-low cell lines are enriched with stem cell-like properties and EMT genes signature *(Prat, 2011)*.

The CSC hypothesis

Historically, it has been believed that random events causing mutations in any somatic cell will give rise to clonally propagated tumors and that all derived cells within the tumor were equally tumorigenic. In recent years, several lines of evidence have converged to support the notion that not all cancer cells within a tumor are equal for tumor initiating potential. In fact, a small sub population of cancer cells exhibiting the ability to self-renew and to regenerate the phenotypic heterogeneity of the tumor was identified and termed cancer stem cells (CSCs), because of the distinctive properties that have been typically attributed to normal stem cells. These CSCs have thus been entailed both in initiating and sustaining primary tumor growth and then in driving the spreading and the growth of metastases *(Al-Haji, 2003; Sheridan, 2006; May 2011)*. In human breast cancer, CSCs were initially identified by the $CD44^{+}/CD24^{-/low}$ antigenic phenotype. Cancer cells displaying $CD44^{+}/CD24^{-/low}$ population have capability to self-renew, to reconstitute the heterogeneity of the tumor and are able to grow in culture as mammospheres *(Al-Haji, 2003)*, additionally, CSCs can form the tumor at a much higher frequency than the bulk of the tumor when injected in NOD/SCID mice in limiting dilution assay *(Wright, 2008)*.
Additional markers were also recognized to enrich CSC populations, including aldehyde dehydrogenase (ALDH1) (Ginestier, 2007) and CD133 (prominin-1) *(Wright, 2008)*. Notably, these molecules are not expressed universally across all types of breast CSCs, but rather are differentially expressed, probably in relation to subtype or microenvironment alterations *(Charafe-Jauffret, 2009)*.

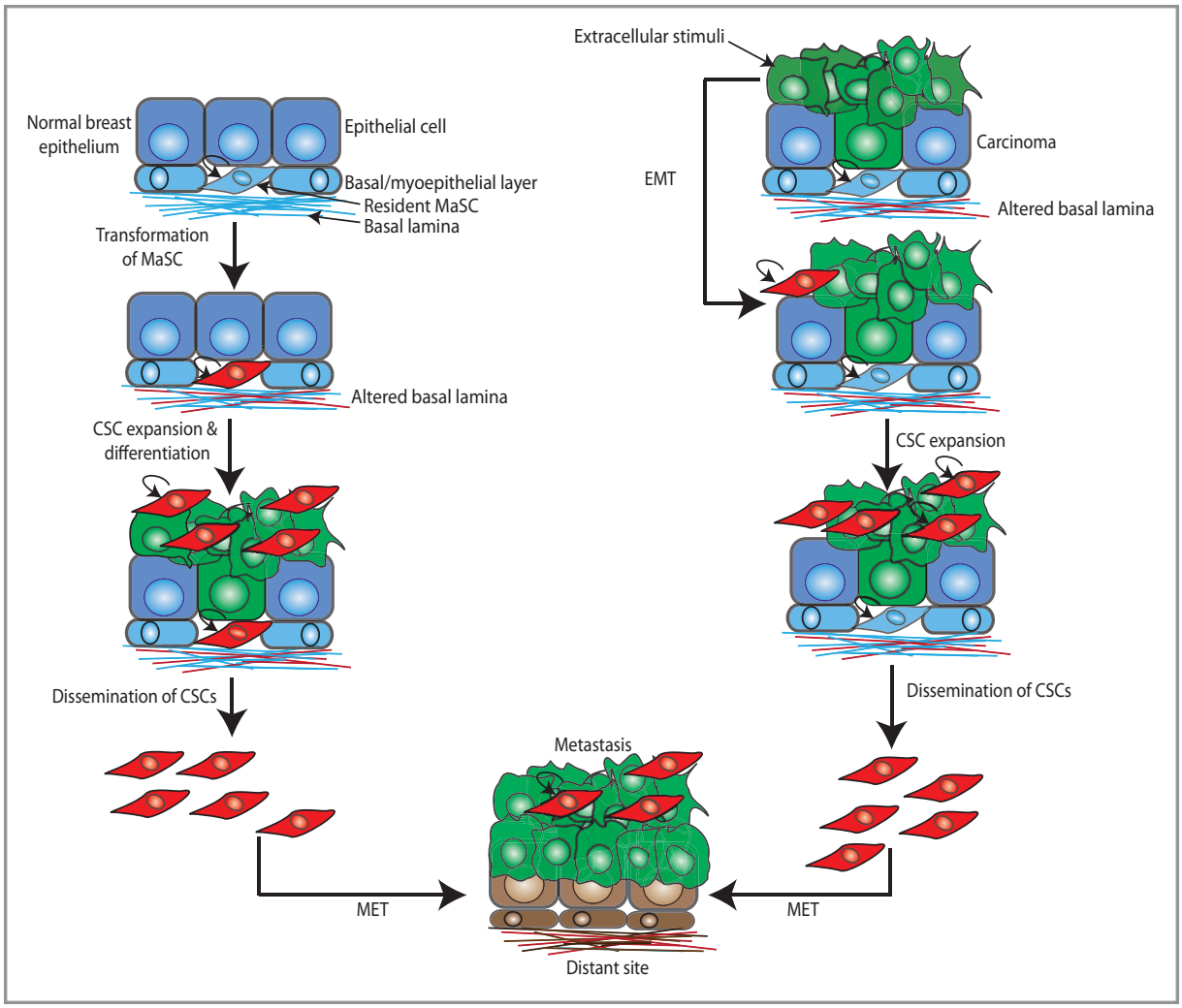


Fig 1 EMT and stem cell traits in breast cancer progression: two overlapping hypothesis.
Breast tumors may originate from the transformation of normal adult tissue stem cells or progenitors that have acquired self-renewal capabilities (left panel). Additionally, the induction of sporadic EMT within a tumor promotes migratory and invasive potential conferring cancer cells with self-renewal capabilities and generating cancer stem cells (CSCs) (right panel). At the metastatic site following extravasation, CSCs (in red) may at least partially revert to an epithelial phenotype (mesenchymal-epithelial transition (MET)) to allow adhesion and proliferation at distal sites.
(adapted from *May, 2011)*

Dissecting breast cancer heterogeneity

Several hypotheses have been proposed to explain the origin of heterogeneity in breast cancer. From a biological perspective, intrinsic breast tumor subtypes might reflect different levels of development of the epithelial normal breast *(Lim, 2009)*. Accordingly, luminal and HER2+ tumors may originate from the transformation of luminal lineage-committed progenitors,

whereas basal-like arise from less differentiated stem cell-like cells. The claudin-low group represent, instead, the undifferentiated tumors that are the most similar to the mammary stem cell. However, there are evidence that luminal progenitors may also serve as precursors to basal-like tumors following genetic or epigenetic events that switch cellular phenotypes, such as loss of BRCA1 or PTEN *(Santarosa, 2011; Polyak, 2011)*.

Expression of epithelial cell adhesion molecule (EpCAM) and CD49f (α6 integrin) have been used to identify luminal and basal cells from normal human mammary tissues. Mature luminal cells are reported to express an $EpCAM^{+}/CD49f^{-}$ phenotype, while luminal progenitors exhibit a $EpCAM^{+}/CD49f^{+}$ profile. Myo-epithelial cells and basal progenitors are defined by the expression of $EpCAM^{-}/CD49f^{+/high}$ population *(Lim, 2009; Keller, 2010)*. However, a univocal phenotype able to discriminate for stem progenitor cells is still lacking. In fact, it has been reported that $EpCAM^{+}/CD49f^{+}$ phenotype may characterize bipotent cells in human tissues *(Villadsen, 2007)*. Given these ambiguities, it has suggested that no unique set of cell surface markers can currently discriminate progenitors from mature cells. Recent studies strongly imply that the majority of human breast cancer are likely derived from $EpCAM^{+}$ luminal epithelial cells, because $EpCAM^{+}$ cells are able to give rise to both ER^{+} and ER^{-} tumors indicating that basal-like tumors do not need to originate from basal/myoepithelial progenitors *(Keller, 2011)*. Indeed, most basal-like tumors were reported to be negative for the well established basal/myo-epithelial marker CD10. Conversely, basal/myo-epithelial cells positive for CD10 are considered to be the source of rare types of breast tumors such as metaplastic tumors *(Keller, 2011)*, the breast cancer most closely related to claudin-low subset *(Hennessy, 2009)*.

Expression of $CD44^{+}/CD24^{-}$ have also been used to discriminate luminal epithelial cells expressing genes involved in hormone responses ($CD24^{+}$) and cells resembling progenitors ($CD44^{+}$) (*Shipitsin, 2007)*.

Further studies are required to determine the cell of origin for each subtype, although it is likely that a different combination of alteration events and cell types transformation may occur within different subtypes *(Prat, 2011)*.

According to the variability of stem markers observed, understanding the origin of breast CSCs is still matter of controversy. Current experimental evidence supports two different but not exclusive theories (**Fig 1**). The first theory proposes that CSCs derive from deregulation of normal stem cell pathways, and in particular from basal mammary progenitors as suggested by the high similarity between normal stem cell and CSCs *(Al-Haji, 2003; Ginestier, 2007)*. The

second theory, instead, ascribes the origin of CSCs to cells that have undergone EMT since they become more prone to aberrant transformation and display many features close to stem cells. This is supported by the related observation that $CD44^{+}/CD24^{-/low}$ cells isolated from human breast tissues express many genes involved in EMT *(Mani, 2008)*.

CSCs: an explanation for tumor recurrence

The finding that CSCc exist has significant implications in breast cancer treatment *(Li, 2008)*. Considering that CSCs are believed to be both the root and the lifeblood of the tumor, these cells need to be eradicated to prevent the tumor propagation. Unfortunately, available treatment strategies are able to hit the bulk of the tumor cells but not CSCs, which can escape death and thus lead to disease recurrence. In fact, there is an enrichment in $CD44^{+}/CD24^{-/low}$ cells in primary breast tumors after conventional chemo- and radio-therapy *(Li, 2008; Phillips, 2006; Ryan, 2011; Velasco-Velazquez, 2012)*. Furthermore, it was observed that this cell population, when transplanted in an *in vivo* mouse model, were capable of self-renewal, suggesting that they truly play important roles in tumor relapse and metastases *(Lagadec, 2010; Takebe, 2011)*.
A number of genes and signaling pathways have been identified as regulators of stem properties, such as self-renewal and pluripotency. Several evidences suggest that cancer stem cells share molecular players with embryonic stem (ES) cells including SOX2, NANOG and OCT3/4/POU5F1. Their ectopic expression, along with LIN28, was shown to be capable to induce a pluripotent phenotype in human somatic cells *(Wu, 2009)*. Other embryonic signaling pathways, such as Notch, Wnt, Hedgehog and transforming growth factor (TGF-β), are essential for stem signaling during embryogenesis *(Ben-Porath, 2008)*. These pathways play critical roles in normal tissue development and maintenance, and are also involved in tight regulation of EMT, whereas deregulation of their signaling have been widely reported in human cancers, including breast *(Takebe, 2011)*. Furthermore, it has been found that CSC gene signatures are highly expressed in the claudin-low and metaplastic tumors another rare subtype of breast cancer *(Li, 2008; Hennessy, 2009)*. Both these tumors are aggressive, highly resistant to therapy and share the hallmarks of EMT *(Dave, 2012)*.
Taken together, the findings that a CSCs population with EMT traits, enhanced metastatic competence and intrinsic therapy resistance exists, suggest that CSC and EMT are related phenomena, that can be exploited to develop functional targeted treatments.

Epithelial-to-Mesenchymal transition features

EMT is a developmental process by which the epithelial cells aquired a mesenchymal phenotype. EMT is a conserved genetic program essential during embryogenesis and in adult tissue repair and maintenance. Deregulation of EMT has been observed in diverse disease, including tumors; in this context, EMT could be considered a critical step in complex progression of metastases *(Thiery, 2009)*. While the significance of physiological function of EMT is well-established, its particular role in tumor progression and metastasis is still under investigation. During this finely tuned process, epithelial cells lose intercellular junctions and cell-matrix adhesion, reorganize cytoskeleton and acquire mesenchymal markers such as vimentin and migratory and invasive capabilities (**Fig 2**) *(Kalluri, 2009)*.

EMT is induced by a plethora of factors belonging to TGF-β, FGF and Notch signaling pathways, which converge to activate transcription factors including members of the SNAI, TWIST and ZEB families. EMT-driving genes act, in part, through transcriptional repression of epithelial-related genes, and in particular E-cadherin gene, which loss is considered an hallmark of EMT induction in cancer. The functional loss of E-cadherin allows tumor cells to lose their epithelial constraints and to acquire a high degree of motility. Thus, E-cadherin expression is subjected to various control mechanisms, including transcriptional repression through E-box elements in the E-cadherin promoter, inactivating mutations and epigenetic modulation through promoter hypermethylation.

However, EMT is a reversible process: at the metastatic site, in the absence of additional EMT microenvironmental stimuli, invasive cells appear to revert to the original epithelial phenotype via mesenchymal-to-epithelial transition (MET) in order to stably root a secondary tumor in a distal site. This hints that accomplishment of metastases probably requires both EMT and MET (**Fig 1)** *(May, 2011; Guttilla, 2011)*.

Along with enhanced migration, EMT has been linked with the acquisition of stem cell features; actually, recent studies demonstrated that the induction of EMT in human mammary epithelial cells resulted in the generation of stem-like cells, in term of ability to self-renew and initiate tumor. The activation of EMT can be driven by TGF-β1 stimulation and either by ectopic expression of EMT–driving factors, among which SNAI1 or TWIST *(Mani, 2008)*, or knocking-down expression of E-cadherin *(Chao, 2010)*. Gene expression pattern of such transited cells resulted very similar to the gene signature found in claudin-low and metaplastic breast cancers.

These data support a direct link between EMT, loss of epithelial properties and gain of CSCs-like properties. Consistently, $CD44^{+}/CD24^{-/low}$ populations isolated from human breast cancer specimens cells show an EMT gene expression signature *(Creighton, 2010; May, 2011)*.

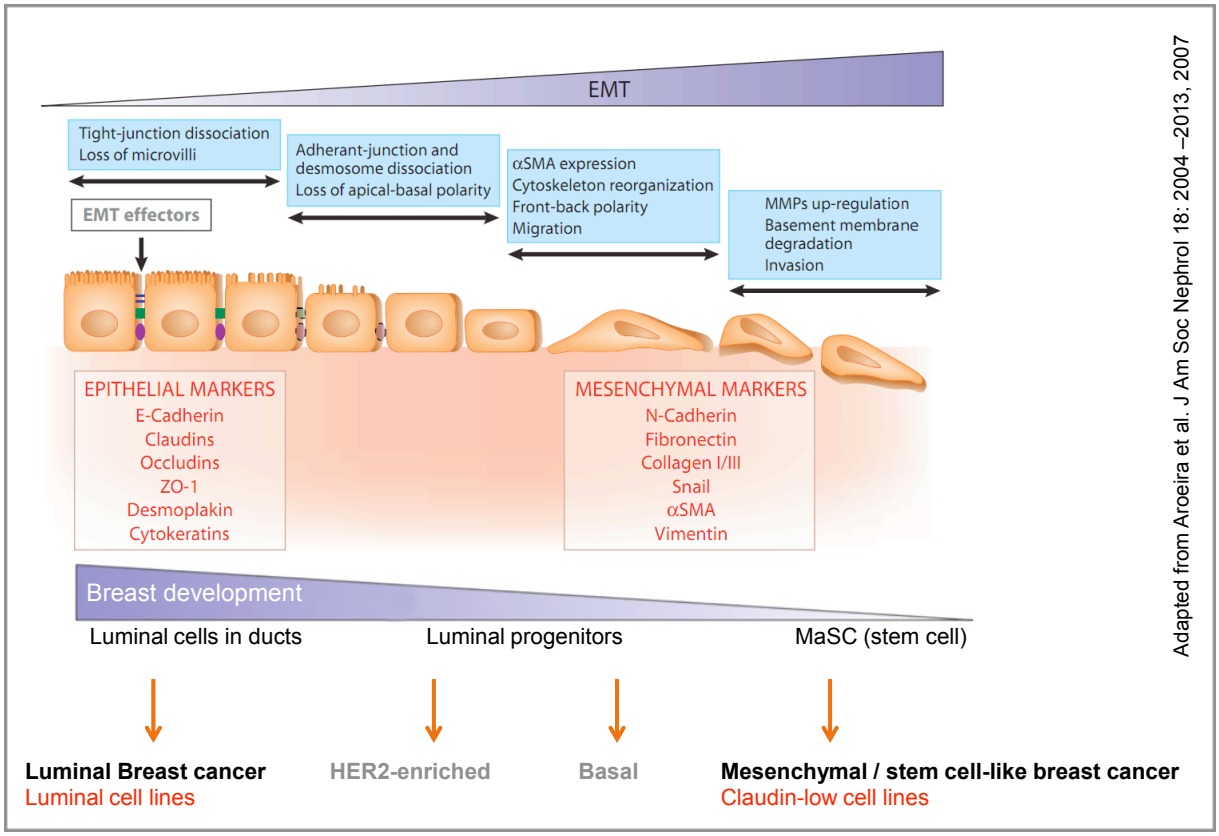


Fig 2 Key events during EMT are related to breast cancer heterogeneity.
The diagram shows four key steps that are essential for the completion of the entire EMT course and the pivotal genes that are modulated during this process *(Aroeira, 2007)*. The various breast tumor subtypes are molecularly compared to subpopulations from normal breast tissue and the different steps of EMT. Each cancer subtype may represent a stage of developmental arrest for a tumor with an origin earlier in the differentiation hierarchy or, alternatively, transformation of a cell type at one specific stage of development.
(adaptation *by S. Tugendreich, IPA Ingenuity System)*

Much effort has focused on understanding the signals which conferred EMT. However, the consequences of EMT on cell survival and response to therapeutics are still poor clarified. Recently, a study pointed out the role of EMT in promoting resistance to EGFR (Epidermal Growth Factor Receptor) inhibition in multiple carcinoma types. These results appear paradoxical considering that EGFR signaling promote cell self-renewal, while chronic activation of EGFR in epithelial-derived cancers promotes EMT-like transition and tumor progression. Nevertheless, it was hypothesized that these signals, once EMT has occurred, become

redundant and are replaced with survival networks to maintain the mesenchymal-like state, even if the EGFR signaling apparatus remain intact. This gradual regulation of EGFR can be explained by the hypothesis that the EMT could be a continuum of three but distinct states: a pre- or potential EMT state, a reversible "metastable" and an "epigenetically-fixed" EMT state. In this frame, these dynamic changes can be considered as the result of a heterogeneous remodeling of the cells during tumor progression *(Thomson, 2011)*.

Regulating EMT

Cancer cells metastasizing from the tumor bulk are frequently characterized by EMT phenomena *(Brabletz, 2005; Schmalhofer, 2009)*. Hypoxia and several stimuli derived by stroma or inflammatory compartment may trigger the expression of a plethora of EMT-transcription factors *(Polyak, 2009; May, 2011)*. As reported above, these factors were originally identified as transcriptional repressors of E-cadherin, because of their ability to directly or indirectly bind to conserved E-boxes sequences in the proximal promoter of this gene *(Thiery, 2009; Moreno-Bueno, 2008)*. EMT-driving factors include, amongst all, the family members TWIST (TWIST1 and TWIST2), members of the SNAI family (SNAI1/SNAIL and SNAI2/SLUG) and ZEB factors (ZEB1 and ZEB2/SIP1) *(Schmalhofer, 2009)*.

Zinc-finger-homeodomain proteins ZEB1 and ZEB2 are complex transcription factors that exert their functions in multiple developmental pathways during embryogenesis, whether mutations in ZEB encoding genes cause severe syndromic malformations *(Vandewalle, 2009)*. Enforced expression of ZEB factors in epithelial cells results in a rapid EMT associated with a breakdown of cell polarity, loss of cell-cell adhesion and induction of cell motility *(Eger, 2005; Aigner, 2007)*. Moreover, ZEB1 promotes metastases in a mouse xenograft model, indicating a role for ZEB1 in invasion and metastases of human tumors *(Spaderna, 2008)*. Vice versa, knockdown of ZEB factors in undifferentiated cells induces a mesenchymal-to-epithelial transition (MET). If aberrantly over expressed, these potent factors contribute to malignant progression of various epithelial tumors. Actually, their aberrant expression has been reported in many human cancer types including breast, ovarian, gastric and oral squamos cell carcinomas , where it is a crucial activator *(Vandewalle, 2009; Browne, 2010; Brabletz, 2010)*, in particular, at the invasive front of several cancers. In breast, its expression was found to be mainly restricted to triple negative breast cancers *(Graham, 2009)*.

Intriguingly, evidence suggests that ZEB1 couples activation of EMT and maintenance of stemness *(Wellner, 2009)* by modulating the small non coding RNAs, namely microRNAs, miR200c *(Gregory, 2008)*, whose members are strong inducers of epithelial differentiation and have a stemness inhibiting function. MicroRNAs act by inhibiting gene expression at the post-transcriptional level and are emerging as master regulators of differentiation *(Wright, 2010)*. Recent studies have reported that ZEB1 and miR200c are reciprocally regulated in a negative feedback loop, each strictly controlling the exclusive expression of the other *(Brabletz, 2010)*. Concordantly with ZEB factors expression in tumors, miR200c has been seldom detected in basal-like type tumors and its reduced expression was observed in metastases compared to primary tumor *(Burk, 2008; Gregory, 2008; Iliopoulos, 2009)*. ZEB1 and miR200c feedback loop has been proposed as a regulator of cellular plasticity in development and disease, and in particular as a driving force for cancer progression towards metastases by controlling the state of cancer stem cells *(Brabletz, 2010)*.

SNAI family members are highly conserved zinc finger transcription repressors implicated in the early embryonic developmental processes and in human cancer their expression was observed in the invasive carcinoma cells. In breast cancer, the expression of SNAI1 and SNAI2 are associated with tumor recurrence and poor prognosis, but whereas expression levels of SNAI1 correlates with the loss of E-cadherin and metastases, SNAI2 expression associates with a partially differentiated phenotype although high levels are detected in basal-like breast cancer (*Peinado, 2007)*. Also SNAI factors are negatively controlled by a direct post-transcriptional regulation by miRNAs (miR124a) *(Lee, 2010)*, which exclusive expression was confirmed also in different cell lines *(Sreekumar, 2011)*.
Both SNAI1 and SNAI2 share common target genes with the other EMT-driving genes, such as epithelial genes including citokeratins CK17/18, CK19 and claudin-1,-3, -4 ,-7, and also EMT-driving genes themselves as occurs for the SNAI1-TWIST1 axis in breast cancer *(Peinado, 2007; Foubert, 2010)*.

TWIST proteins are helix-loop-helix factors that play an important role in neural crest formation during embryogenesis. Similar to ZEB and SNAI family, they are potent inducers of EMT and markers of stem cell status. Over expression of TWIST1 has been reported in multiple solid tumors and is associated with early metastases and poor clinical outcome *(Ansieau, 2010; Sreekumar, 2011)*.

Finally, accumulating evidence supports the idea that a multistep relationship between the co-expression of key EMT molecules and poor cancer prognosis exist. In this model, SNAI1 and ZEB2 play a role in activating the first EMT steps that induce cells to invade, whereas SNAI2 and ZEB1 favor the maintenance of the migratory, invasive phenotype, and TWIST1 has a critical role in the development of distant metastases by prompting cells to enter the bloodstream *(Foubert, 2010)*.

RNA-interference library screen

RNA-interference screen has recently become a powerful tool for the identification of critical genes in cells. One way to identify such genes is the inhibition of their expression by RNA interference (RNAi) followed by the analysis of the resulting "loss-of-function" phenotype. New generation shRNA libraries contain vectors each expressing one shRNA coupled with a unique barcode sequence, allowing the identification of shRNA that produce a specific phenotype in complex pools. Barcode is an additional shRNA-associated sequence that increases the sensitivity and the robustness of the screen (*Silva, 2008; Root, 2006; Boettcher, 2010; Chang, 2006)*. Once defined the effective readout of "loss-of-function" phenotype in cell models, the shRNA library-based screen can be a very useful high-throughput approach to identify important genes involved in tumor progression.

AIMS

The hypothesis underlying this study is that common determinants of both EMT and stemness exist and may control the metastatic potential of breast tumors. Thus, the understanding of molecular mechanisms underpinning EMT and CSC phenotype in breast cancer may lead to the development of targeted treatments for patients that currently do not benefit from an effective therapy.

On this ground, we consider to:

1-Investigate the modulation of EMT and stem molecular signature by stem enriching culture conditions in a series of breast cancer cell lines.

We present data on characterization of a panel of ten breast cancer cell lines in term of mesenchymal, epithelial and stem signatures in standard and in three-dimensional culture condition.

2-Evaluate the effect of EMT-driver knock down on EMT and stem features.

The loss-of-function experiments allow us to shed light on the role of the known EMT drivers in the control of stem potential and therapy response. Furthermore, this provide information about the markers mainly modulated in the EMT and stem reversion to use as a readouts in the sorting EM- and stem-reverted cells.

3- Identify novel molecular regulators of both EMT and CSC phenotypes.

To this end, we design a high throughput screen based on shRNA-library technology that allows us to recognize genes whose silencing result in MET and loss of stem properties in breast cancer model. This screen is performed in collaboration with Greg Hannon (Cold Spring Harbor, NY). Given the complexity of this reverse genetic screen, within the time frame of the PhD, we consider to accomplish the setting-up phase.

RESULTS

1. CELL LINE MODELS RECAPITULATE EMT AND STEM ENRICHED TUMORS

Data reported in this chapter have been the subject of a submitted publication: *Borgna et al, Mesenchymal and stem features are induced in mammospheres obtained from breast cancer cell lines*

Mesenchymal and luminal features in cell lines grown in adherence and in anchorage independent stem conditions

Several studies have shown that breast cancer cell lines similar to primary breast tumors may be classified as luminal A or B, basal-like and claudin-low/mesenchymal. Among the different markers that distinguished these groups of cells, E-cadherin, vimentin and luminal/basal markers as well as transcription factors regulating EMT are differentially expressed between luminal, basal and claudin-low cell lines. These differences were obviously confirmed in the panel of ten cell lines used in this study, representing basal-like/claudin-low tumors (HBL100, MDA157, MDA231, MDA436, Hs578T), basal A subtype (MDA468), HER2$^+$ tumor (SKBR3) and luminal group (BT474, MCF7, T47D).

In fact, mesenchymal markers were mainly expressed by the claudin-low cells, previously described as basal B or mesenchymal-like *(Neve, 2006; Blick, 2008; Hollestelle, 2009)*, whereas the epithelial E-cadherin characterized luminal cell lines (**Fig 1**).

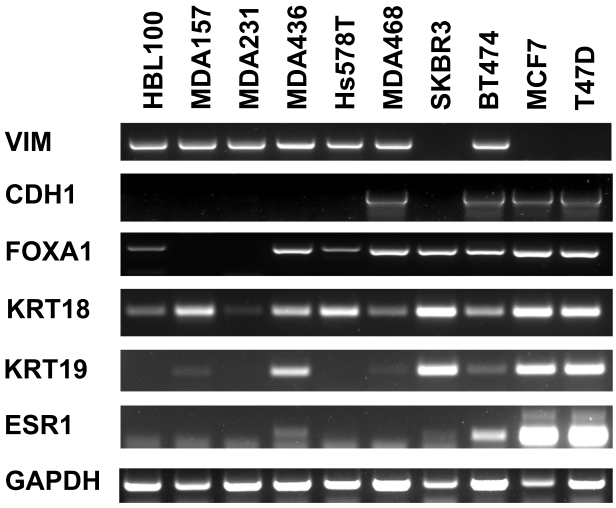


Fig 1 - Mesenchymal and epithelial markers in breast cancer cell lines

Detection of mesenchymal markers vimentin (VIM), FOXA1 and epithelial markers E-cadherin (CDH1), citokeratin-18, -19 (KRT18 and KRT19) and estrogen receptor (ESR1) by RT-PCR.

GAPDH was reported as control.

Consistently, high levels of EMT-associated transcription factors ZEB1, ZEB2, SNAI1/SNAIL, SNAI2/SLUG and TWIST1 were detected particularly in the claudin-low lines. Accordingly with the complex interaction among EMT-drivers, each claudin-low cell line expressed a number of the known EMT-drivers. ZEB2 was marked expressed in all 4 claudin-low cell lines tested (Fig 2 -B), whereas TWIST1 expression appeared to be not restricted to claudin-low cells, because of its expression also in BT474 luminal B cell line (**Fig 2**A).

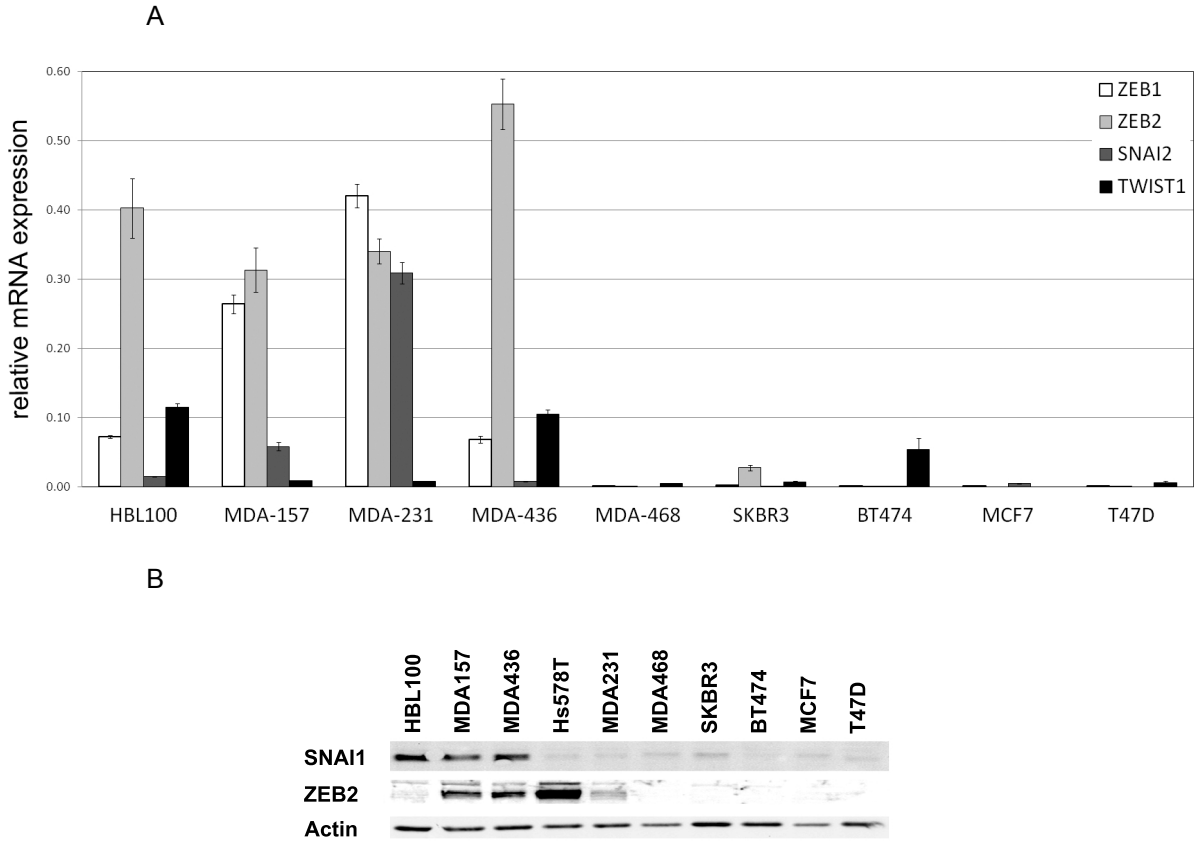


Fig 2 - Mesenchymal drivers in breast cancer cell lines

Detection of EMT- transcription factors ZEB1, ZEB2, SNAI2, TWIST (**A**) by qRT-PCR and SNAI1 and ZEB2 (**B**) by Western Blot.

Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S. Actin was used as loading control in Western Blot. Bars, SD.

Conversely, miR200c, a non coding RNA able to regulate gene expression by targeting ZEB1/2 and SNAI2 mRNAs, was distinctly expressed in luminal cell lines (**Fig 3**).

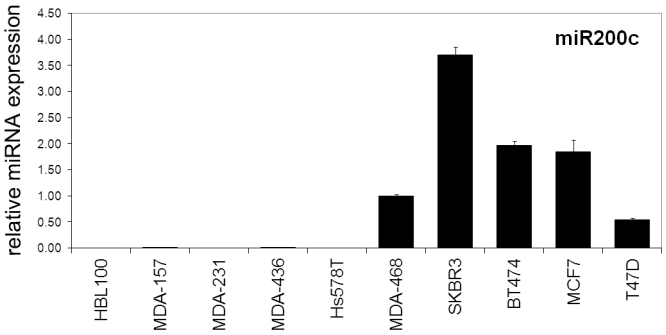


Fig 3 - miRNA expression in breast cancer cell lines

Expression of epithelial driver miR200c by qRT-PCR.

Relative expression of miR200c was normalized to RNU48. Bars, SD.

Mammosphere culture has been utilise to enrich for normal adult stem cells and for CSCs (*Charafe-Jauffret, 2009; Dontu, 2003)*. Since both mammosphere culture and EMT expand the CSC fraction and due to the connection between EMT and CSCs, we examined whether non adherent 3D culture could induce significantly alterations in EMT-related genes expression. Interestingly, epithelial and mesenchymal markers as well as EMT transcription factors are modulated in cells grown in 3D cultures.

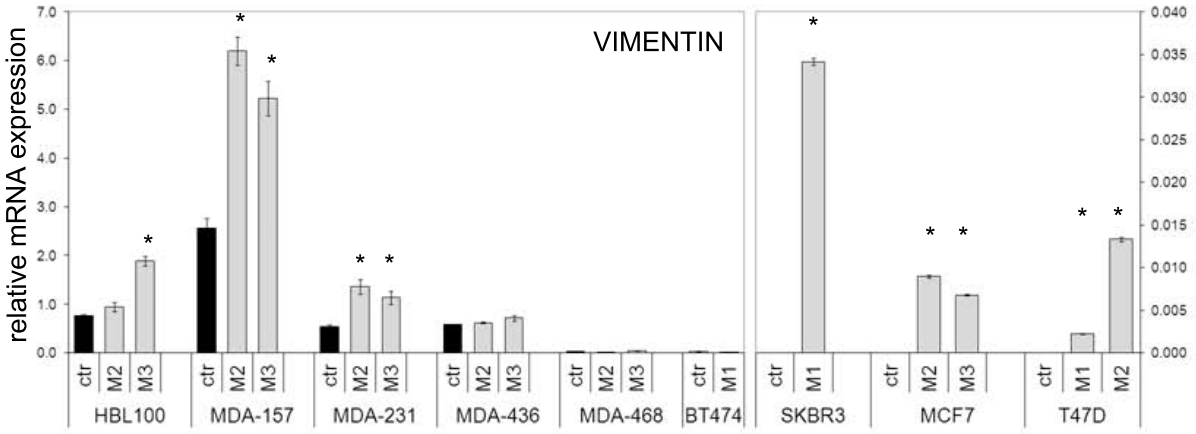


Fig 4 - Vimentin modulation in 3D culture conditions

Detection of mesenchymal marker vimentin in all cell lines by qRT-PCR.

Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S.
M1, M2 and M3 refer to primary, secondary and tertiary mammospheres in stem-medium.
Ctrl refer to cells grown in standard medium conditions. *p<0,05

In fact, a noticeable increased in mesenchymal marker vimentin (**Fig 4**) and a diminution of the epithelial markers CDH1, KRT18, KRT19 and ESR1 in luminal cell lines have been detected (**Fig 5**A-D). Moreover, the ERα gene ESR1 expression decrease in the luminal cells. Interestingly, in both MCF7 and BT474 cell lines grown as mammospheres, we observed an augment in cells expressing CD10 molecule, a surface marker of myo-epithelial cells *(Schenka, 2008)* (**Fig 6**).

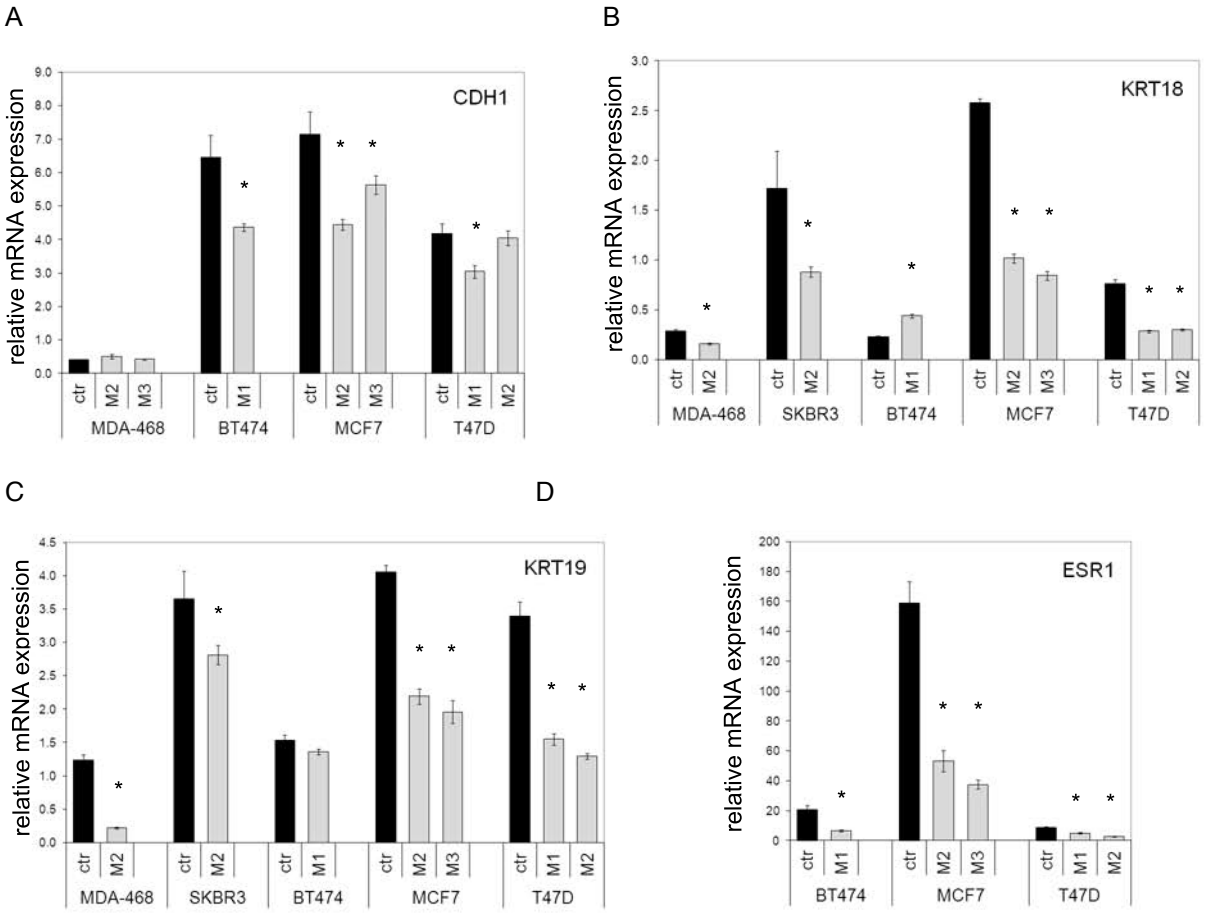


Fig 5 - Epithelial markers modulation in 3D culture conditions

Detection of epithelial E-cadherin (CDH1) (**A**), citokeratin-18 (KRT18) (**B**), citokeratin-19 (KRT19) (**C**) and ESR1 (**D**) in luminal and HER2$^+$ cell lines by qRT-PCR.

Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S.
M1, M2 and M3 refer to primary, secondary and tertiary mammospheres in stem-medium.
Ctrl refer to cells grown in standard medium conditions. *p<0,05

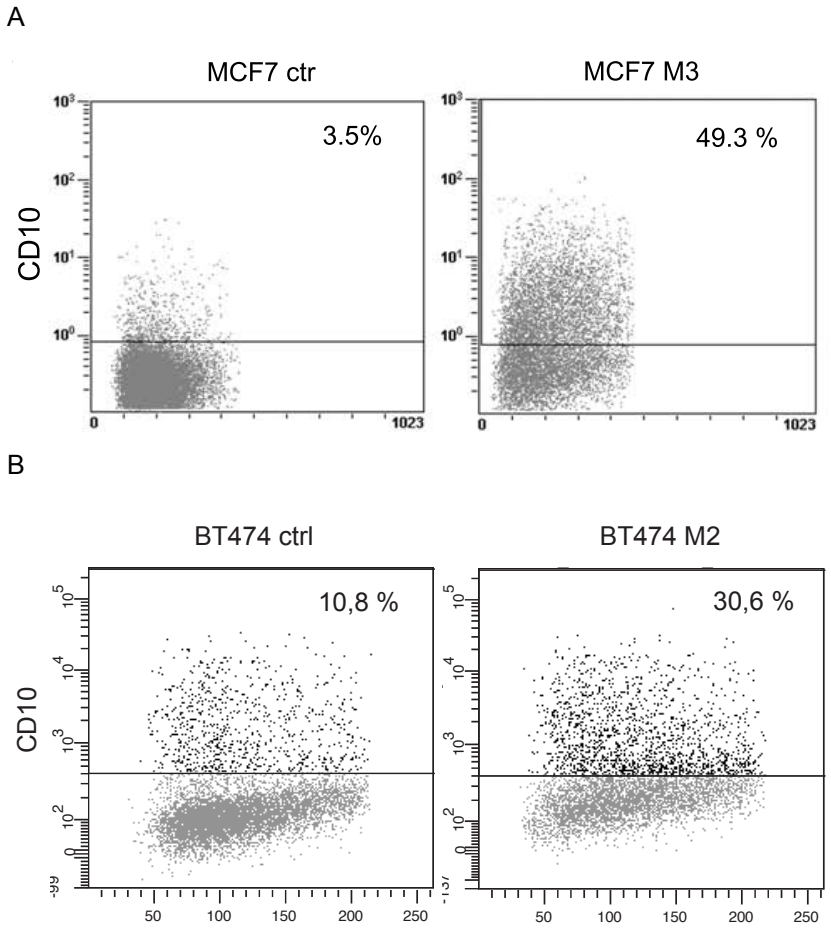


Fig 6 - Myo-epithelial marker modulation in 3D culture conditions

Flow cytometry analysis detecting CD10 in MCF7 and MCF7-M3 mammospheres (**A**) and BT474 and BT474-M2 mammospheres (**B**).

M2 and M3 refer to secondary and tertiary mammospheres. Ctrl refer to cells grown in standard condition for MCF7 or in adherence with stem-medium condition for BT474. Gates for positive cells set on isotype control.

Similarly, we analyzed expression levels of EMT-driver genes. The mesenchymal features associated with augment in different TFs in each cell lines (**Fig 7**A-D). Interestingly, TWIST1 was the only transcription factor that significantly increase in all but MDA157 lines grown as mammospheres including luminal cells (**Fig 7**D). Furthermore, a significant ZEB1 increase was observed in MDA157 and in MDA436 cell lines (**Fig 7**A), ZEB2 in MDA231 and in the tertiary spheres of MDA436 (M3), whereas SNAI2 increment was detected exclusively in the HBL100 cell line (**Fig 7**C).

Taken together these data suggests that mesenchymal phenotype induced by 3D culture condition may be stimulated by a combination of transcription factors, and TWIST1 above all.

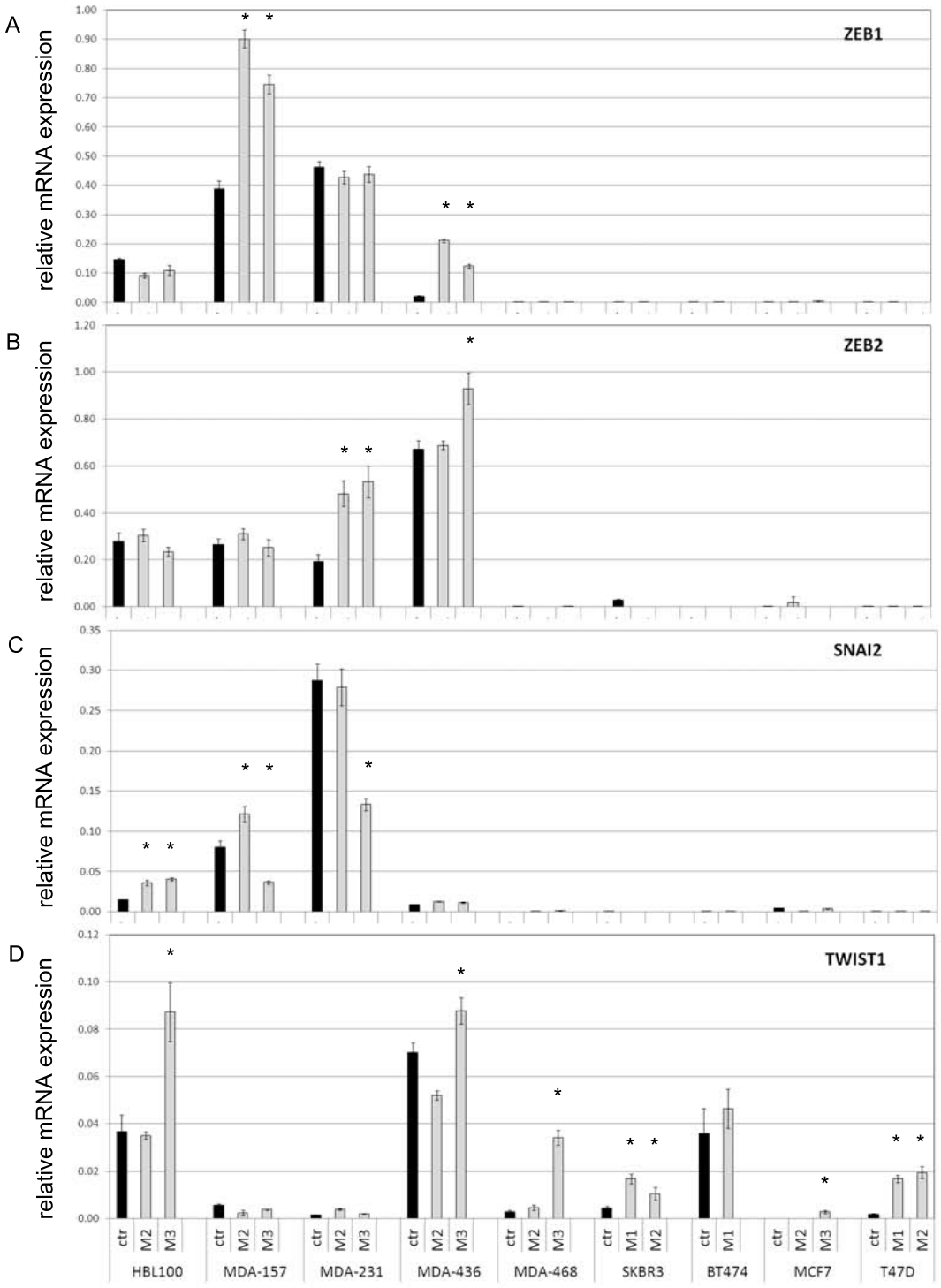


Fig 7 - EMT-transcription factors modulation in 3D culture conditions

Detection of ZEB1 (**A**), ZEB2 (**B**), SNAI2 (**C**) and TWIST1 (**D**) in cells grown in 3D culture by qRT-PCR.

Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S.
M1, M2 and M3 refer to primary, secondary and tertiary mammospheres in stem-medium.
Ctrl refer to cells grown in standard medium conditions. *p<0,05

Stem population were differentially expressed in breast cancer cell lines

$CD44^{+}/CD24^{-/low}$ antigenic profile defines cells with CSCs features *(Al Haji, 2003)*. The same cells have been reported to express a mesenchymal phenotype *(Blick, 2010)*. Actually, $CD44^{+}/CD24^{-/low}$ cells were more common in basal-like tumors. The surface marker CD133 can also isolate a group of breast cancer stem cells that does not overlap with $CD44^{+}/CD24^{-/low}$ *(Verslues, 2009)*.

We observed that all but HBL100 claudin-low cell lines showed a more then 90% of cells expressing the $CD44^{+}/CD24^{-/low}$ phenotype (**Fig 8**A). In HBL100 cell line both $CD44^{+}/CD24^{-/low}$ and CD133 positive profiles were represented whereas MDA468 cells expressed mainly CD133 antigen but not the $CD44^{+}/CD24^{-/low}$ surface profile (**Fig 8**B). Luminal and HER2 cells were entirely enriched with CD24 positive cells and negative CD133 population, resulting in the absence both of $CD44^{+}/CD24^{-/low}$ (**Tab1**).

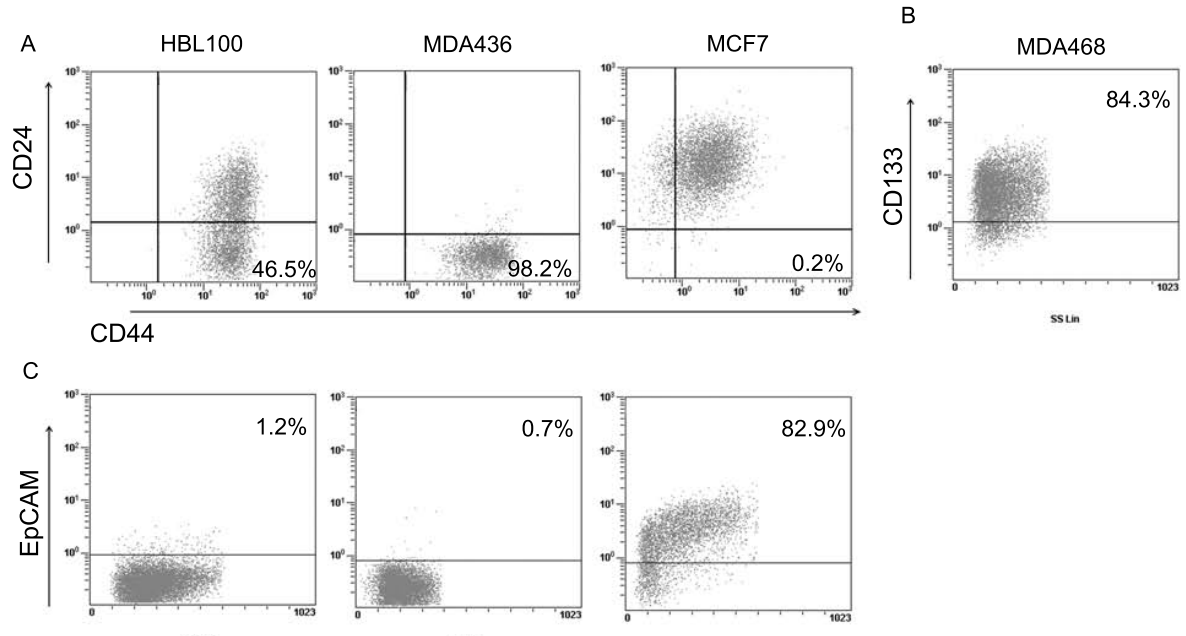


Fig 8 - Antigenic profile population accounts for stem properties
Representative flow cytometry analysis detecting $CD44^{+}/CD24^{-/low}$ (**A**) and EpCAM (**C**). Positive population in MDA436, HBL100 and MCF7 cell lines. CD133 antigen detection in MDA468 cells (**B**). Gates for positive cells set on isotype control.

However, culture cells as mammosphere was not sufficient to induce an increase of $CD44^{+}/CD24^{-/low}$ population in all but SKBR3 luminal cells. In fact, in SKBR3 CD24 was partially down regulated whereas CD44 increased (**Fig 9**A). Surprisingly, in MCF7 cells grown as

mammospheres CD44 decreased (**Fig 9**B). Finally, the mammary lineage marker EpCAM represented at least 70% of the luminal cell populations and of the basal-like cells (MDA468). The percentage of EpCAM positive cells varied in claudin-low cells from very low levels (HBL100, MDA436, Hs578T) to 15-40% of MDA157 and MDA231, respectively.

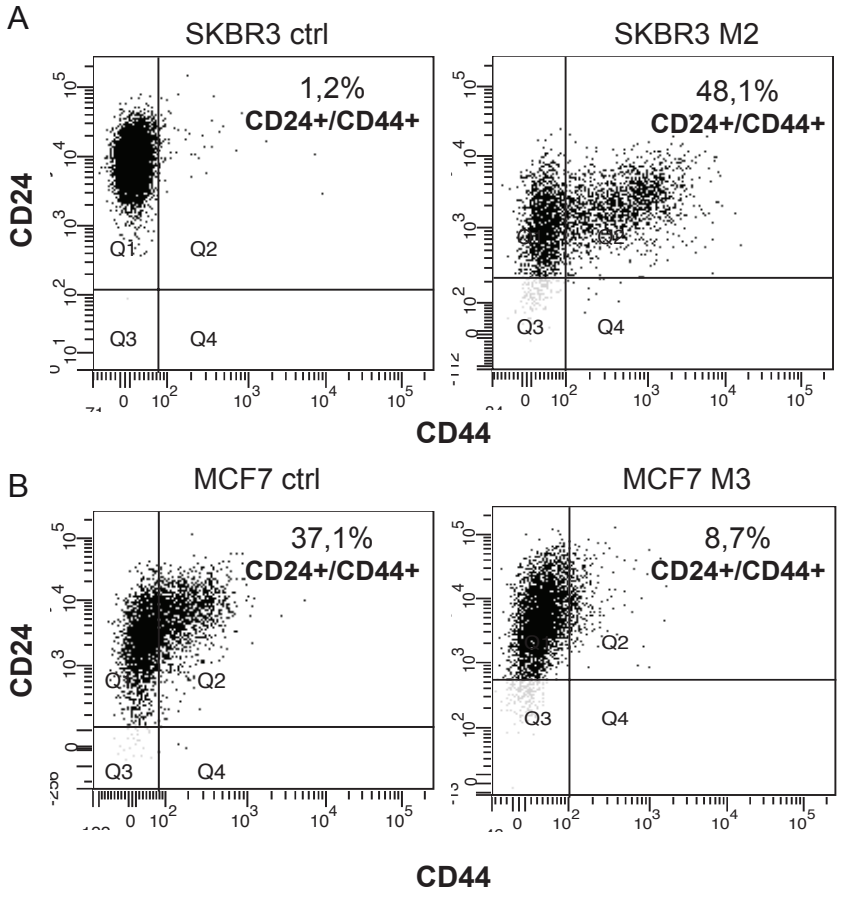


Fig 9 - CD44 but not CD24 is affected by 3D culture

Flow cytometry analysis detecting CD24 and CD44 in SKBR3 and SKBR3-M2 (**A**) and MCF7 and MCF7-M3 mammospheres (**B**).

M2 and M3 refer to secondary and tertiary mammospheres. Ctrl refer to cells grown in standard conditions. Gates for positive cells set on isotype control.

Cell lines	Tumor subtype	% CD24	% CD44	% $CD44^+/CD24^-$	% CD133	% EpCAM
HBL100	Claudin-low	50 ± 15	99 ± 1	50 ± 10	10 ± 1	2 ± 1
MDA157	Claudin-low	1 ± 1	98 ± 2	98 ± 2	1 ± 1	15 ± 3
MDA231	Claudin-low	3 ± 3	98 ± 2	97 ± 3	1 ± 1	41 ± 16
MDA436	Claudin-low	8 ± 8	98 ± 2	95 ± 5	2 ± 1	1 ± 1
HS578T	Claudin-low	1 ± 1	98 ± 2	98 ± 2	2 ± 1	1 ± 1
MDA468	Basal-like	98 ± 2	98 ± 2	2 ± 1	80 ± 4	80
SKBR3	HER2	99 ± 1	5 ± 2	0	2 ± 1	90
BT474	Luminal	99 ± 1	1 ± 1	0	1 ± 1	75
MCF7	Luminal	97 ± 2	92 ± 5	0	2 ± 1	72 ± 12
T47D	Luminal	97 ± 2	5 ± 2	0	2 ± 1	86 ± 11

Table 1 - Stem cell populations in breast cancer cell lines.

Apart from flow cytometry analysis of stem cell markers, the ability of tumor cells to form mammospheres as surrogate assay of the stemness of the cells has been described. Surprisingly, we observed that the ability to grow as mammospheres was not limited and related to the presence of $CD44^+/CD24^{-/low}$ phenotype. In fact, among the cell lines tested, we detected a high sphere forming efficiency (SFE) in luminal MCF7 cells lacking $CD44^+/CD24^{-/low}$ population and, conversely, low SFE in the almost $CD44^+/CD24^{-/low}$ pure cell line MDA231 (**Fig 10**A,B). Therefore, more than one antigenic profile might characterise distinct stem/progenitor cells.

Elevated levels of aldheyde deydrogenase (ALDH1), an enzyme involved in retinoic acid metabolism, has been associated with poor prognosis in breast cancer and used to enrich cells with CSC-like features *(Ginestier, 2007; Charafe-Jauffret 2009; Charafe-Jauffret 2010)*. Recently, ALDH1A3 isoform was recognised as a novel CSCs marker in contrast to the previously identified ALDH1A1 *(Marcato, 2011)*. Our findings correlate with the hypothesis depicting ALDH1A3 isoform as marker of CSCs instead of ALDH1A1, as revealed by its expression in all but Hs578T claudin-low cell lines (**Fig 10**C).

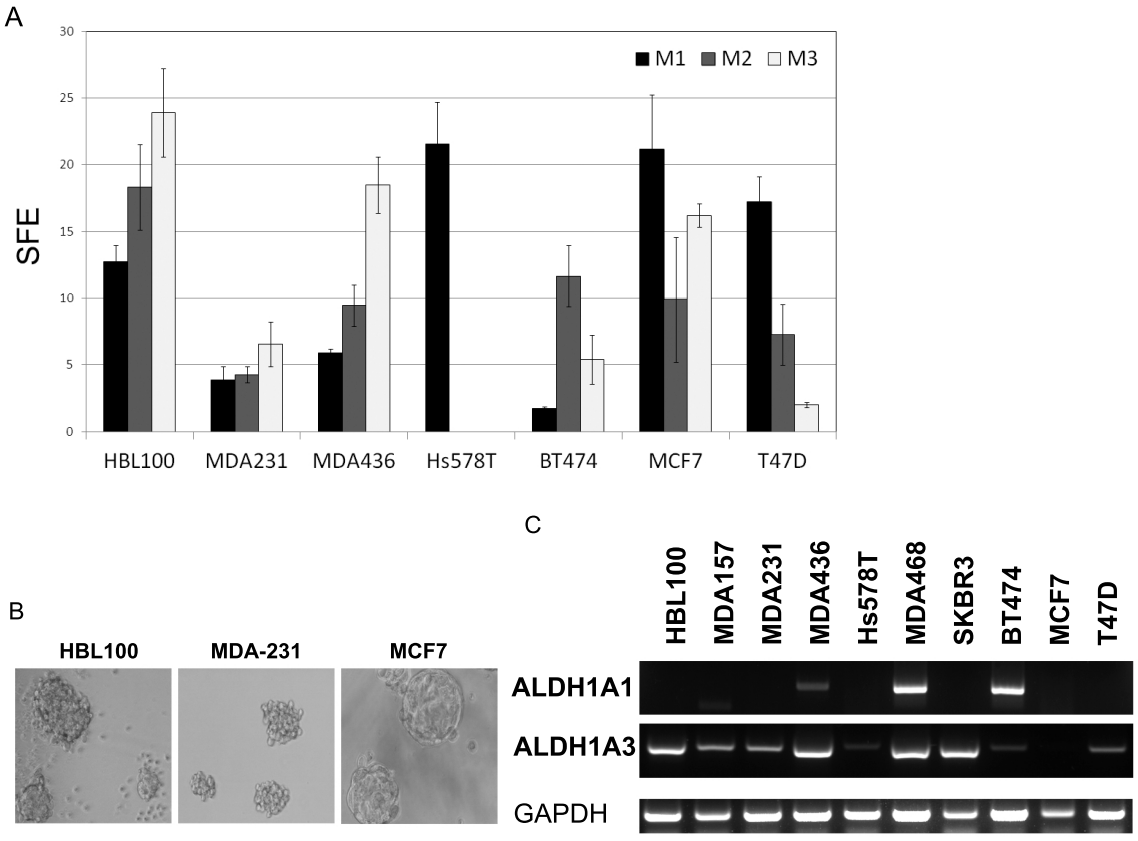


Fig 10 - Sphere forming efficiency (SFE) and stem-factors expression account for stem properties

Different mammosphere forming ability in claudin-low and luminal cell lines. Sphere forming efficiency (SFE) is reported. (**A**) Representative images of spheres obtained from MDA231, HBL100 and MCF7. (**B**) RT-PCR of the isoform ALDH1A1 and isoform ALDH1A1 putative stem markers in the breast cancer cell lines.

M1, M2 and M3 refer to primary, secondary and tertiary mammospheres.
Photos were taken at 100x magnification.
GAPDH was reported as control.

Mounting evidence suggests that embryonic stem genes and signaling pathways, among which SOX2, POUSF1, Nanog and Notch respectively, were reactivated in cancer in order to stimulate tumors to gain CSCs *(Ben-Porath, 2008; Takebe, 2011)*. To shed light on the involvement of these embryonal signaling pathways in breast cancer we measured the expression of SOX2, POUSF1, Nanog, NOTCH1 and its target HEY1 in the ten cell lines. Intriguingly, only MCF7 cell line expressed SOX2 (**Fig 11**). We failed to observe POU5F1 (OCT3/4), Nanog expression in any of the ten cell lines tested although a positive control, such as the embryonal carcinoma NTera-2D1 (NT2D1) cell line, was used (data not shown). Again, the $CD44^{+}/CD24^{-/low}$ profile

did not correlate with the expression of these stem genes suggesting that other pathways might be involved in the staminal features of the mesenchymal CSC. The levels of Notch receptor did not show a direct relationship with claudin-low and luminal clusters. However, its target HEY1 was mainly expressed in luminal and in the basal A cell lines.

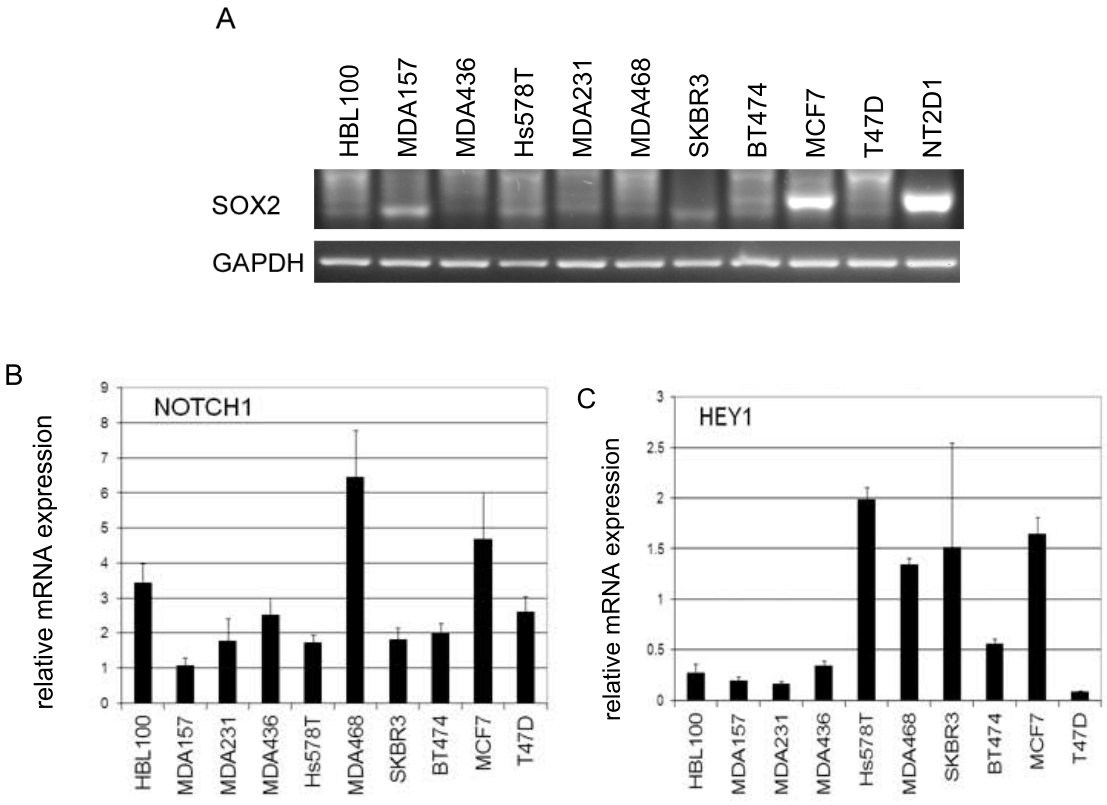


Fig 11 - Stem factors in adherent conditions

Detection of stem factors SOX2 by RT-PCR (**A**), Notch1 (**B**) and HEY1 (**C**) in cells grown in adherent culture by qRT-PCR.

GAPDH was used loading control in RT-PCR. Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S. Bars, SD.

Three dimensional stem cultures stimulated a distinct increase in SOX2 protein levels in MCF7 cell line (**Fig 13**A) and mainly affected Notch1 target HEY1 levels rather than the expression of its receptor (**Fig 13**B,C).

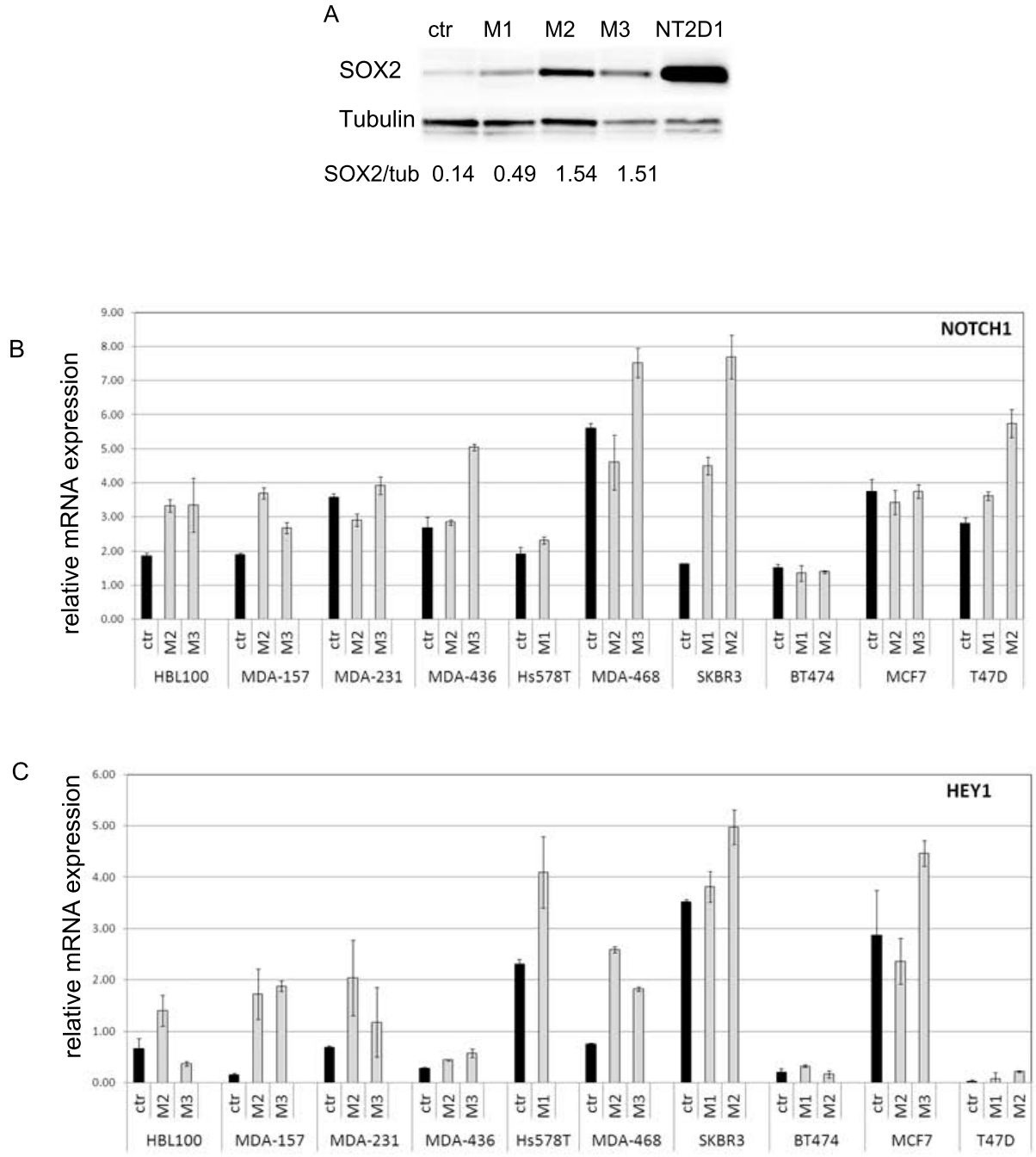


Fig 13 Stem factors stimulation in 3D culture conditions

Increasing of SOX2 expression by western Blot (**A**) in MCF7 mammospheres. Detection of NOTCH1 (**B**) and HEY1 (**C**) in cells grown in 3D culture by qRT-PCR.

Tubulin was used loading control in Western Blot and quantitative ratio is reported.
Relative expression of transcripts analysed by qRT-PCR were normalized to three references gene: β-2-microglobulin, β-actin and 18S. Bars, SD.
M1, M2 and M3 refer to primary, secondary and tertiary mammospheres. Ctrl refer to cells grown in stem-medium conditions.

2. *EFFECT OF EMT-DRIVER KNOCK DOWN ON EMT AND STEM FEATURES*

Data reported in this chapter have been the subject of a submitted publication:
Borgna et al, ZEB1 and aberrant DNA methylation sustain mesenchymal features, stem phenotypes and EGFR inhibitor resistance in breast cancer.

ZEB1 and SNAI2 down regulation partially reverts the mesenchymal-like phenotype

The characterization of breast cancer cell lines arising from different tumors subtypes allowed us to select best models in which perform the shRNA screen, in order to detect a reduction in mesenchymal/stem properties and a gain of epithelial/differentiated phenotype. Among tested cell lines, the claudin-low MDA231 and MDA157 cell lines turned out to display a clear mesenchymal phenotype, EMT-transcription factors expression and CSCs markers. We focused on these cells, and in particular on the highly-invasive MDA231, as a model for our screen.
One of the major inducers of EMT is ZEB1. Its expression has been demonstrated to strongly associate with impairment of E-cadherin and other epithelial markers. Indeed, as reported above, MDA231 and MDA157 expressed high levels of ZEB1 in combination with ZEB2 and SNAI2 (in MDA231) and mesenchymal features. The expression of more than one EMT-determinant was central in our models to recapitulate the redundant network of EMT-driving genes typical of breast tumor. To test whether silencing a single EMT-transcription factor resulted in MET and stem reversion, we first knock down ZEB1 in MDA231 and MDA157 cell lines by lentiviral delivery. Depletion of ZEB1, achieved with all three shRNA sequences used (**Fig 14**A,B), caused a conversion of cellular morphology from a spindle-like to a cobblestone-like shape, typical of luminal differentiated cancer cells, particularly in MDA231 (**Fig 14**C). This morphological change is supported at molecular level by the increased expression of luminal cytokeratins -18 and -19 and restoration of claudins -3 and -4 (**Fig 12**C-E). In spite of this, the phenotype was not fully reverted from mesenchymal to epithelial, but resulted in a slight re-activation of E-cadherin only in MDA231 (**Fig 13**D) and a weak reduction of the mesenchymal marker vimentin (**Fig 13**F).

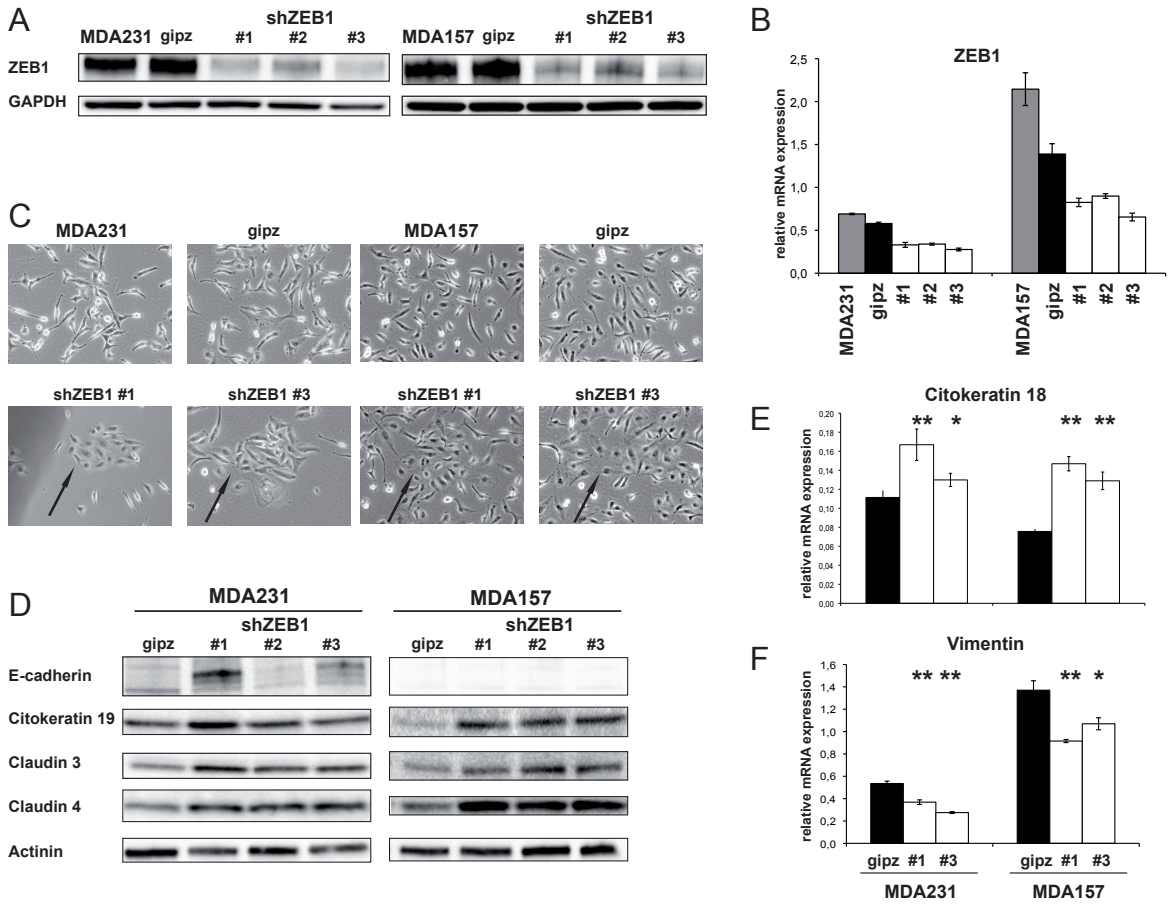


Fig 13 - ZEB1 silencing partially reverted EMT phenotype

Knock down of ZEB1 was performed in MDA231 and MDA157 cell lines by shRNA infection with three lentivirus vectors. Expression level of ZEB1 was detected by Western Blot (**A**) and qRT-PCR (**B**). Cells acquired a cobblestone morphology upon down regulation of ZEB1 (**C**) compared to control infected and parental cells; arrows indicate cobblestone clusters. Photos were taken at 100x magnification. ZEB1 knock down causes epithelial marker re-expression such as E-cadherin, Citokeratin 19, Claudin-3 and -4 (by Western Blot) (**D**) and Citokeratin 18 (by qRT-PCR)(**E**) and reduction of vimentin (by qRT-PCR)(**F**).

GAPDH and actinin were used loading control in Western Blot.
Relative expression of transcripts analyzed by qRT-PCR were normalized to three references gene as described in Matherial and Methods.
#1, #2 and #3 refer to three different ZEB1 shRNA sequences; gipz refers as scramble infected vector. Bars, SD. Asterisks indicate p values <0.05 (*) or p values <0.01 (**).

It is well established that E-cadherin is frequently silenced in breast cancer through aberrant promoter hypermethylation *(Dumont, 2008)*. To explore whether the scarce expression of E-cadherin in shZEB1 knock down cells was due to methylation of CDH1 promoter, we treated MDA231 and MDA157 cell models with 5 μM of 5′-AZA (5′-azacytidine) for 72 hours (or DMSO as negative control). Noticeable, treatment with demethylating agent strengthened re-expression of E-cadherin in ZEB1 silenced clones of both cell lines whereas it proved ineffective in the ZEB1-expressing controls (**Fig 14**A). To understand whether the epigenetic regulation

might control also the E-cadherin repression mediated by other EMT transcription factors, we knocked down also SNAI2 in the Hs578T cell line. Again, silenced cells did not re-express E-cadherin but after 5′-AZA exposure (**Fig 14**B).

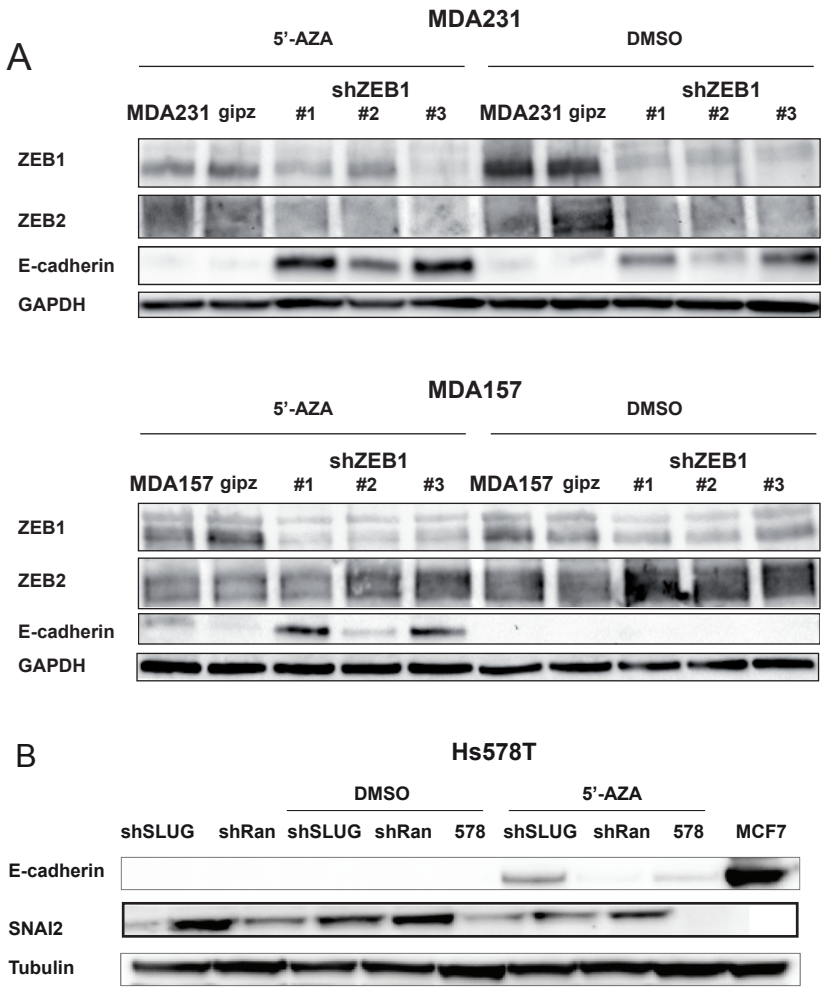


Fig 14 - Demethylating treatment resulted in a marked epithelial transition in ZEB1 and SNAI2 knock down cells

(**A**) Immunoblot showing expression of ZEB1, ZEB2 and E-cadherin in ZEB1 knock down cells treated with 5 μM 5′-azacytidine (5′-AZA) or DMSO as control. (**B**) Knock down of SNAI2 in Hs7578T cells activating E-cadherin only after 5 μM 5′-azacytidine.

MCF7 are used as positive control for E-cadherin.
GAPDH and β-tubulin were used as loading control in Western Blot.

Since ZEB family members and miR200 are reciprocally regulated in a negative feedback loop *(Gregory, 2008; Brabletz, 2010)* and miR200 cluster is regulated by methylation *(Neves, 2010; Davalos, 2011)*, we measured miR200c as a consequence of ZEB1 silencing and after treatment with 5′-AZA.

In MDA231 re-expression of miR200c was detectable after down regulation of ZEB1, whereas no augment of miR200c was observed in ZEB1 silenced MDA157. Once more, de-methylating treatment resulted in a markedly up regulation of miR200c levels in ZEB1 knock down cells and a small re-expression in control cells both in MDA231 and MDA157, which is consistent with the restoration of E-cadherin protein (**Fig 15**). Importantly, whereas the up regulation of miR200c in ZEB1-silenced and de-methylated samples did not result in further ZEB1 down regulation, it caused a down regulation of ZEB2 in 5′-AZA treated cells in both cell lines (**Fig 14**A). All these data suggest that depletion of distinct EMT-drivers is mandatory to obtain a complete reversion of mesenchymal to epithelial phenotype, called MET. This was in line with the observation that MET occurred as a consequence of ectopically expression of miR-200c which in turn resulted in down regulation of ZEB1/2, and SNAI12 (**Fig 16**A,B).

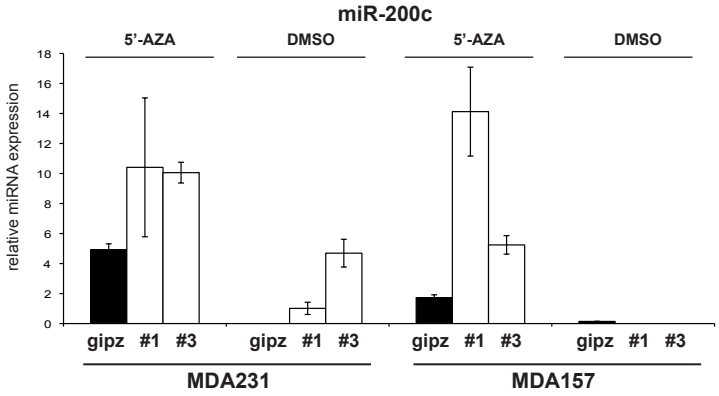


Fig 15 - Demethylating treatment increased re-expression of miR200c in ZEB1 knock down cells
miR-200c expression levels, detected by qRT-PCR, increase after demethylating treatment.
Relative miR-200c were normalized to RNU48. #1, #2 and #3 refer to three different ZEB1 shRNA sequences; gipz refers as scramble infected vector. Bars, SD.

In fact, as well demonstrated in the literature, miR200c is alone efficacious in determining a reduction of its target ZEB1, the mesenchymal marker vimentin and a re-expression of E-cadherin, due to its multiple upstream role in regulating target genes *(Gregory, 2008)*.

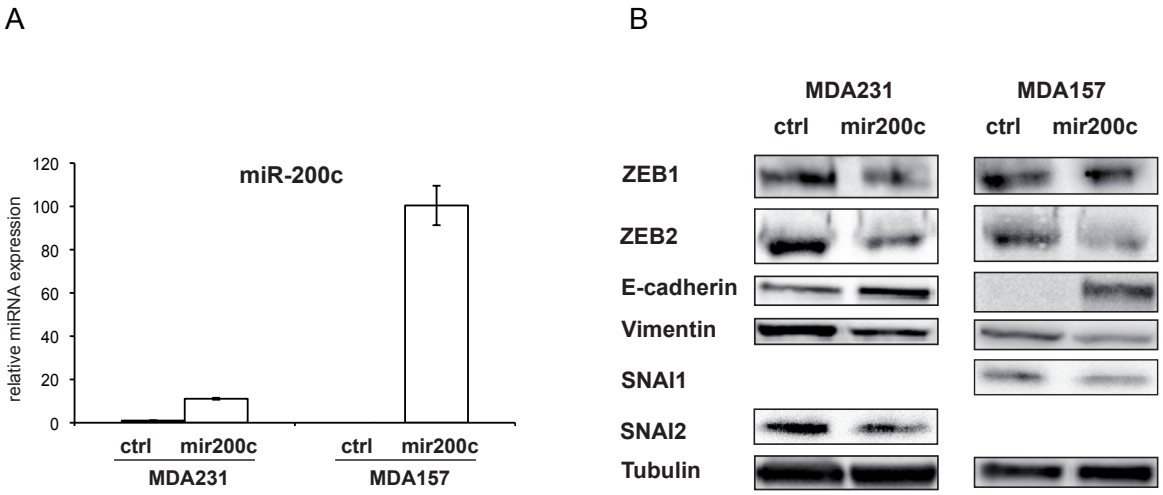


Fig 16 - Expression of miR200c affected mesenchymal markers expression

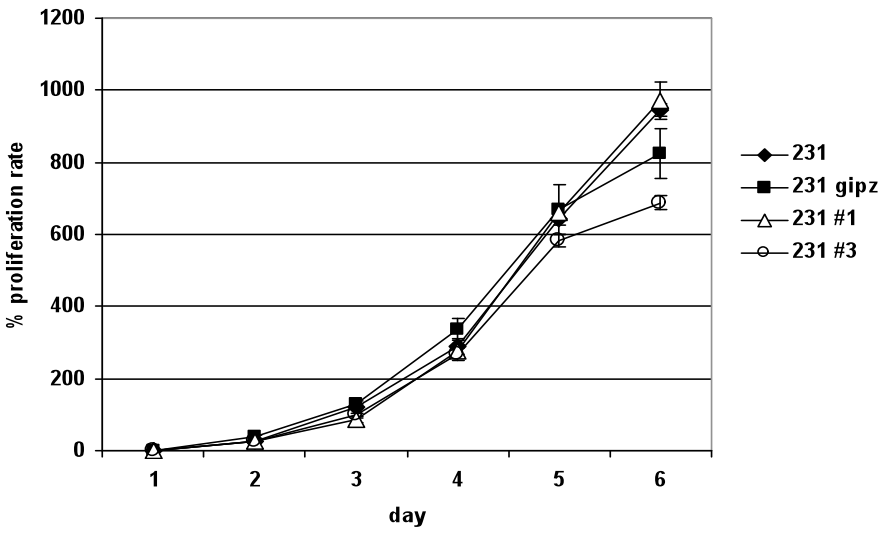
MDA231 and MDA157 stably expressing miR200c compared to scramble vector (qRT-PCR) (**A**). Reduction in ZEB and SNAI members, mesenchymal vimentin and re-activation of E-cadherin in miR-200c over expressing cells (Western Blot) (**B**).

Relative miR-200c were normalized to RNU48. β-tubulin was used as loading control in Western Blot.
#1, #2 and #3 refer to three different ZEB1 shRNA sequences; gipz refers as scramble infected vector. Bars, SD.

Stem population was affected by the down regulation of ZEB1

A functional identification of stem properties bases on the capability of CSC to form mammospheres in non-adherent culture conditions *(Dontu, 2003)*. Thus, we tested the functional ability of our cell models to give rise to mammospheres. In 3D culture, MDA231 cells formed small loosely adherent structure sphere-like, whereas MDA157 gave rise to clusters of cells that survived passaging but could not be considered spheres (data not shown). Therefore, only MDA231 models were investigated for their ability to form primary and secondary mammospheres. Intriguingly, ZEB1 knock down cells, although able to proliferate as the parental cells (**Fig 17**A,B), showed a reduced capability to form spheres which was still more evident in the secondary spheres formation (**Fig 18**A,B).

A



B

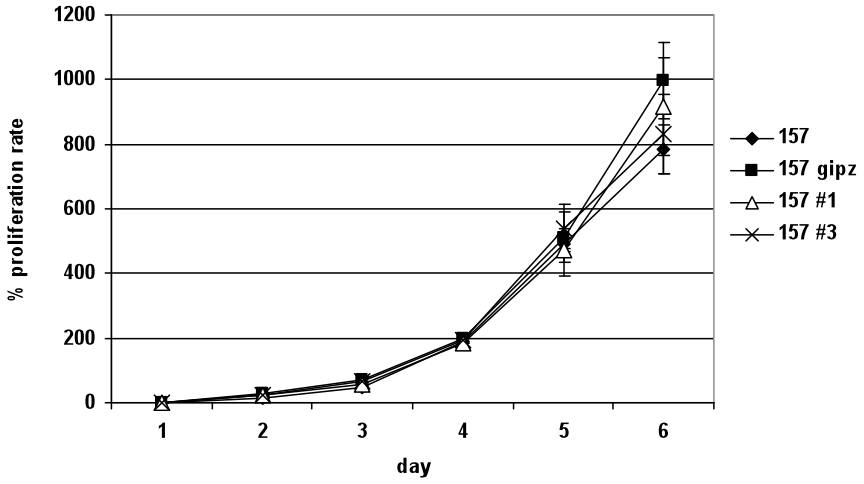


Fig 17 - ZEB1 down regulation did not affect cell proliferation

MDA231 (**A**) and MDA157 (**B**) proliferation rate was measured by SRB assay.

Proliferation rate was calculated as the increment of colorimetric value of cells of a given day (1 to 6) in respect to cells at the day 1. #1 and #3 refer to three different ZEB1 shRNA sequences; gipz refers as scramble infected vector. Bars, SD.

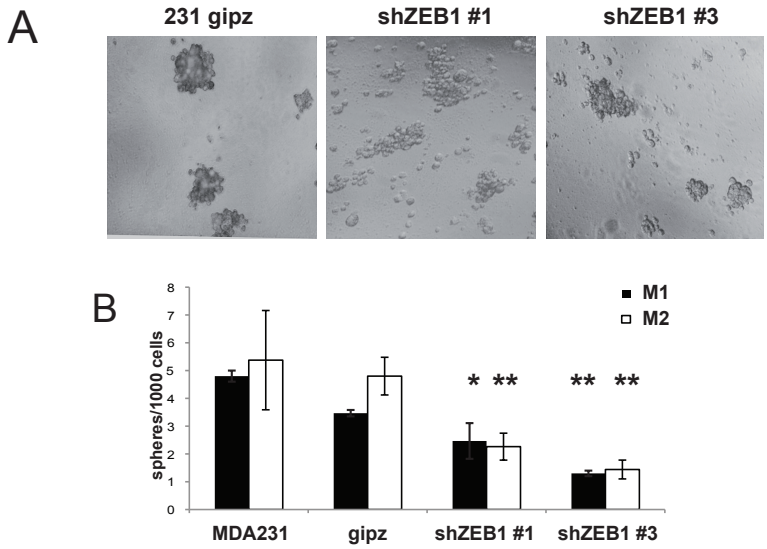


Fig 18 - ZEB1 affects mammospheres formation

Loss of mammosphere forming ability in ZEB1 silenced cells: representative images of spheres obtained from MDA231 gipz, shZEB1 #1 and #3 (**A**); primary (M1) and secondary (M2) spheres of MDA231 cell models growth in 3D. Sphere forming efficiency (SFE) is reported (**B**). Bars, SD.

Photos were taken at 100x magnification.
Asterisks indicate p values <0.05 (*) or p values <0.01 (**).

Claudin-low breast cancers were demonstrated to be statistically enriched for CSCs *(Creighton, 2009)*, that display the cell surface $CD44^{+}/CD24^{-/low}$ profile *(Al-Haji, 2003)*.

In order to gain insight whether ZEB1 has an important role in stemness, in addition to its role in maintaining mesenchymal phenotype, we investigated stem properties in our cell models.

First, the $CD44^{+}/CD24^{-/low}$ phenotype was evaluated in our cell models. Indeed, MDA231 and MDA157 showed an almost totally $CD44^{+}/CD24^{-/low}$ population *(Sheridan, 2006; Wang, 2007; Murohashi, 2010; Hwang-Verslues 2009)*. Importantly, ZEB1 knock down generated cells with CD24 positive phenotype both in MDA231 and MDA157 cell lines (**Fig 19**A,B), whereas CD44 positive population was not affected (data not shown).

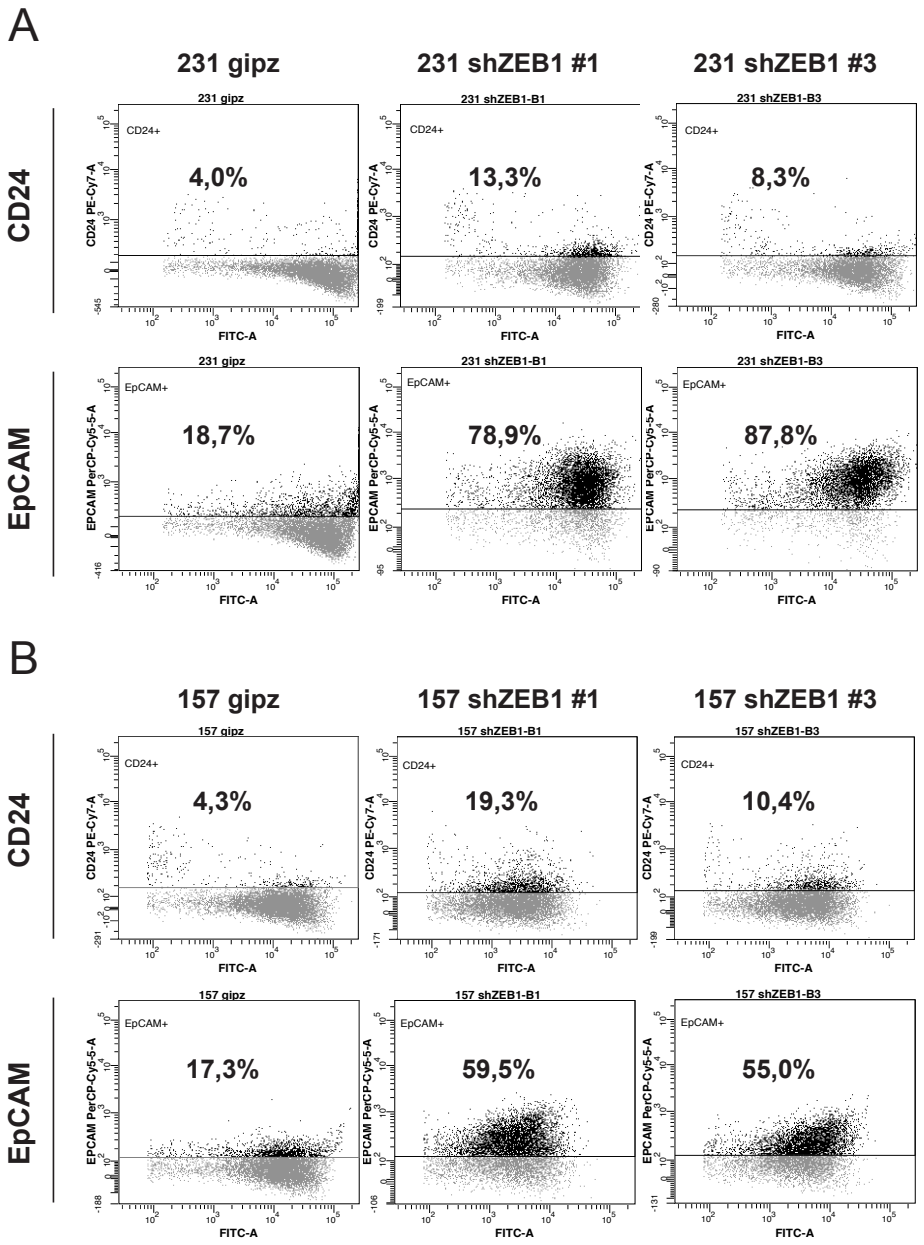


Fig 19 - ZEB1 affected CD24 and EpCAM expression.

Flow cytometry analysis detecting CD24 and EpCAM positive population in ZEB1 knock down cells in MDA231 (**A**) and in MDA157 models (**B**).

Gates for positive cells were set on isotype control. Color compensation by subtracting GFP-FITC channel was performed.

Furthermore, cells expressing epithelial cell adhesion molecule EpCAM, which has been reported to identify progenitor/differentiated luminal cells *(Lim, 2009)*, augmented in ZEB1 silenced clones compared with control cells (**Fig 19**A,B).

Cells treatment with 5'-AZA resulted in a modest increase in CD24 and EpCAM positive populations in both ZEB1-silenced and control MDA231 cells (**Fig 20**A,B). These data point to a

direct effect on promoter demethylation rather than an involvement of the 5'AZA-induced miR200c. Actually, EpCAM has been described to be epigenetically regulated *(Spizzo, 2007)*.

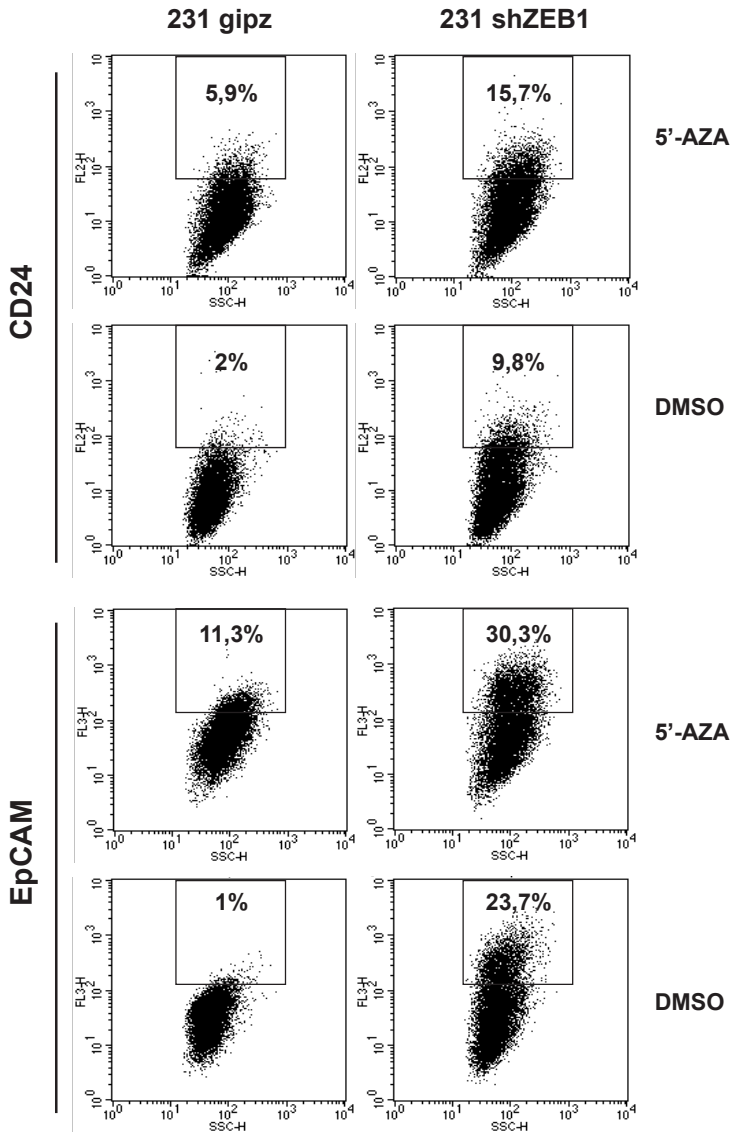


Fig 20 - Demethylation enhanced ZEB1 down regulation effect on CD24 and EpCAM ri-expression in MDA231 cells.

Increase in CD24 and EpCAM population in MDA231 models after demethylating treatment (5'-AZA) compared to control (DMSO).

Gates were set on DMSO treated 231 gipz cells.

The fact that luminal/differentiated markers were not markedly induced by 5'AZA treatment in the ZEB1 silenced cells suggested that ZEB1 might act on stemness independently from miR200c induction or through the modest miR200c induced after its silencing. Consistently,

ectopically expressed miR200c was almost ineffective not only in modulate CD44 population (data not shown) but also in increasing CD24 and EpCAM positive populations (**Fig 21**).

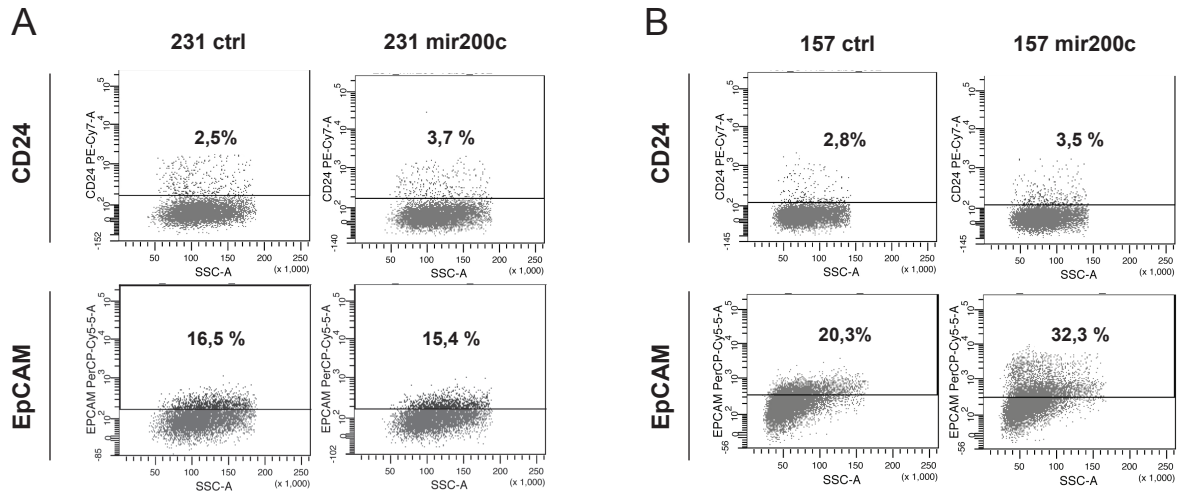


Fig 21 - ZEB1 affected CD24 and EpCAM rather than miR200c expression.
CD24 and EpCAM antigenic profile of miR-200c overexpressing MDA231 (**D**) and MDA157 (**E**) cell lines. Gates for positive cells were set on isotype control.

ZEB1 influenced EGFR activation

Triple negative breast cancers have been reported to frequently express EGF receptor (EGFR) prompting to the use of EGF-targeted agents in triple-negative breast cancer therapies *(Carey, 2011)*. However, clinical studies are controversial probably due to the heterogeneity of patients treated. For example, lacking of information on EGFR status on claudin-low tumors may be a cause of these failures. Furthermore, mounting evidence suggest that EMT can promote resistance to EGFR tyrosine kinase inhibitors in several tumor types expressing this receptor *(Thomson, 2005; Buck, 2007; Haddad, 2009)*.

Here, we evaluated the expression, activation and sensitivity to EGFR of our cell models. Indeed, both MDA157 and MDA231 cells expressed high level of EGFR (**Fig 22**A,B) in contrast to luminal MCF7 cell line (data not shown). ZEB1 silencing resulted in a weakly EGFR up regulation, particularly in MDA231 (**Fig 22**A). Interestingly, activation of EGFR, as demonstrated by phosphorylation of Tyrosine 1068, was achieved even in the absence of specific stimuli (serum starvation) in both MDA231 and MDA157 ZEB1 silenced cells (**Fig 22**C,D). In the

presence of serum or after EGF stimulation parental and silenced cells showed a marked EGFR activation that was still higher in shZEB1 MDA231 (**Fig 22**C,D).

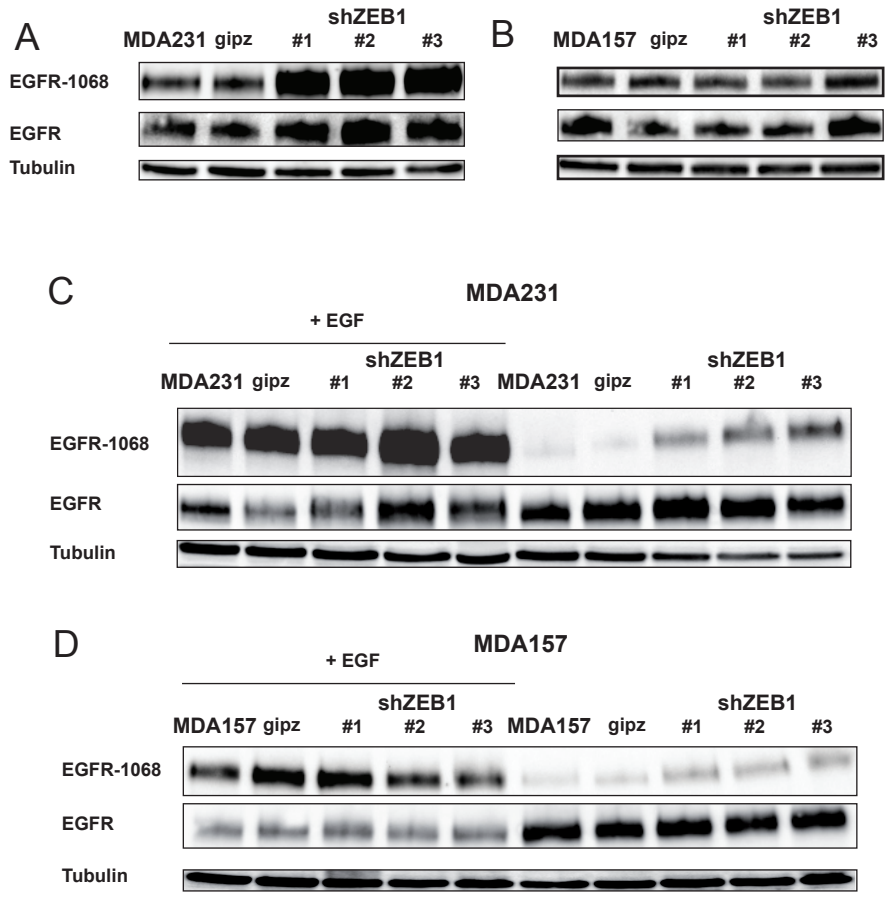


Fig 22 - ZEB1 down regulation enhanced EGFR expression and activation.
Different amounts of EGFR expression in ZEB1 knock down cells (**A**, **B**). Expression and activation of EGFR (immunodetected by phospho-EGFR at Tyr1068) in MDA231 (**C**) and MDA157 (**D**) cellular models cultured in starvation, with or without epidermal growth factor (EGF) stimuli.
β-tubulin were used as loading control in Western Blot.

To investigate whether the EGFR super activation in ZEB1 knock down cells might underline a restored dependence to this proliferative pathway and thus a sensitivity to EGFR inhibitors, MDA231 and MDA157 cell models were challenged with an irreversible EGFR inhibitor (324674). Actually, only MDA231 ZEB1 down regulated cells were slightly sensitive to EGFR inhibitor in the cytotoxic MTT assay (**Fig 23**A), whereas cytotoxic effect on MDA157 models was comparable (**Fig 23**B).

These findings confirmed that EGFR expression was not impaired in EMT *(Thomson, 2011)*. However, EM-transited cells acquired EGFR-independent survival signals and MET restored EGFR-dependence.

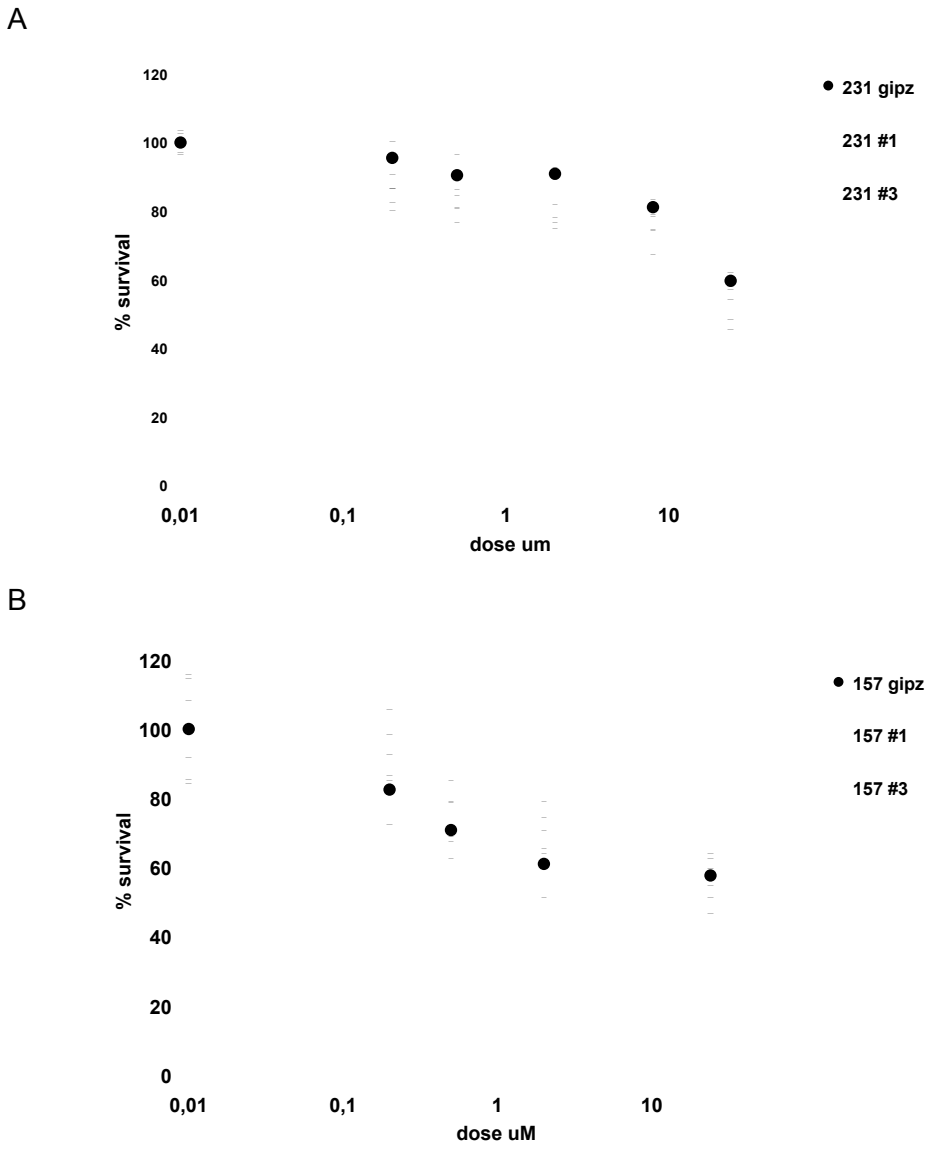


Fig 23 - ZEB1 sensitized cells to EGFR inhibitors treatment.

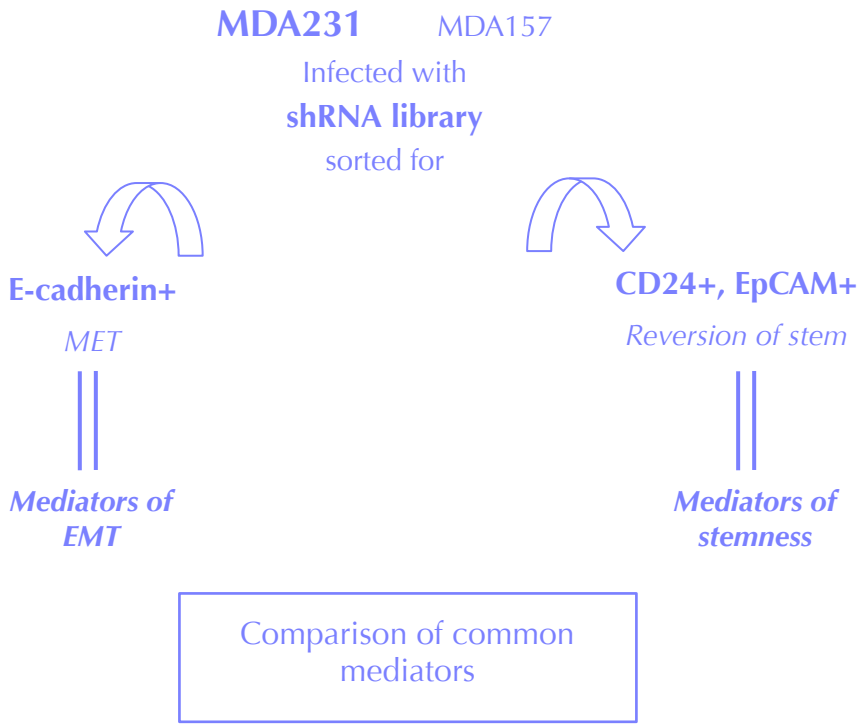
Survival curves of shZEB1 cells and control in MDA231 (**A**) and MDA 157 (**B**) treated with increasing doses of irreversible EGFR inhibitor (324674; 0,2-3,2 μM) assessed by MTT as described in Matherial and Methods.

Surviving fraction represents the mean of three independent expreiments. Bars, SD.
β-tubulin were used as loading control in Western Blot.

3- *IDENTIFICATION OF NOVEL MEDIATORS OF EMT AND CSC*

To identify novel mediators able to induce EMT and CSC, we planned an high throughput shRNA screen with three lentiviral sub-library of 10000 probes in collaboration with Greg Hannon (Cold Spring Harbor, NY) able to identify those genes whose reduction may revert EMT and stem phenotype. The identification of novel regulators of both phenotypes might provide valuable tools for efficient therapies target to eradicate the roots of metastasis, mainly for those patients that do not benefit from any effective therapy.

The flowchart of the shRNA based screening is the following:



The screen based its feasibility on our above reported data that when EMT-driving genes, such as ZEB1 and SNAI2, were silenced in the highly metastatic cell lines MDA231 the reversion of mesenchymal phenotype may be successfully monitored through the re-expression of E-cadherin (**Fig 24**).

Furthermore, in both MDA231 and MDA157, depletion of ZEB1 resulted in the re-expression of CD24 and EpCAM (**Fig 19**A, B).

Thus, in this project we set the experimental conditions by which the screen will be performed. In fact, by flow cytometry we were able to separate both high-positive E-cadherin cells and cells re-expressing EpCAM and CD24 differentiation markers from control cells (gipz) in the selected shZEB1 MDA231. This confirms the feasibility of our sorting system to identify positive MET and to detect the loss of stem features.

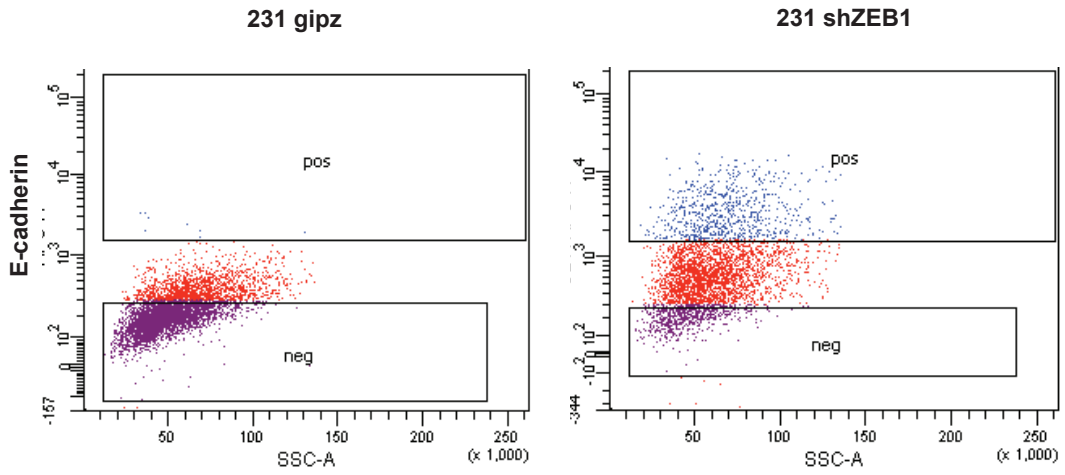


Fig 24 - Sorting system was able to discriminate E-cadherin positive cells after shRNA delivery.
Flow cytometry analysis detecting E-cadherin positive population in ZEB1 knock down cells in MDA231. Gates for positive cells set on isotype gipz control. Color compensation by subtracting GFP-FITC channel was performed. gipz refers as scramble infected vector.

MATERIAL AND METHODS

Cell culture and drug treatments

A panel of ten human breast cancer cell lines (MDAMB-157, MDAMB-231, MDAMB-436, MDAMB-468, MCF7, T47D, BT474, SKBR3) were purchased from the American Type Culture Collection (ATCC) and Interlab Cell Line Collection-Genova (Hs578T, HBL100). All cell lines were validated for short tandem repeat profiling.

MDAMB-157, MDAMB-231, MDAMB-436, MDAMB-468, Hs578T, HBL100, T47D, SKBR3 cell lines were cultured in EMEM (Lonza) supplemented with 10% Fetal bovine serum (Gibco, Invitrogen), non essential amino acids (Gibco, Invitrogen) and antibiotics. MCF7 and BT474 were cultered in RPMI (Lonza) supplemented with 10% FBS and antibiotics.

5′-aza-2′-deoxycytidine (5′-AZA) (Sigma) was dissolved in DMSO and added to the culture medium at 5μM every 12 hours for 72 hours. A corresponding volume of DMSO was added to the untreated cells used as a control. Cells were analyzed after 24 hours from the end of treatment.

MDAMB-157, MDAMB-231, MDAMB-436 and MDAMB-468 are referred as MDA157, MDA231, MDA231, MDA436 and MDA468, respectively.

Generation of stable ZEB1 and SLUG-silenced cells and miR200c over expressing cells

ZEB1-silenced cells (231shZEB1-#1,-#2 and -#3 and 157shZEB1-#1,-#2 and -#3) were obtained from MDA231 and MDA157 cell lines by stable infection with pGIPZ Lentiviral shRNAmir vectors, a kind gift of Prof G. Hannon (Cold Spring Harbour, New York).

Individual sequences of shZEB1 were obtained from Open Biosystem web site, where they are referred as V3LHS_356186 (#1), V3LHS_356184 (#2) and V3LHS_356183 (#3, and validated. Cell lines were transfected with scrambled vector pGIPZ (gipz) as a control.

SLUG-silenced cells (578 shSLUG-A and -B) were obtained from HS578T cell line by stable infection with pLentilox 3.7 vectors, a kind gift of Dr Kylie Niessen (British Columbia Cancer Agency, Vancouver) *(Niessen, 2008)*. Cell lines were transfected with vector plentilox 3.7 containing a random sequence (shRandom) as a control.

Ref. Name		Mature Sense
shZEB1-#1	V3LHS_356186	AGGACAGCACAGTAAATCT
shZEB1-#2	V3LHS_356184	CGGCAAAAGATAGAGAATA
shZEB1-#3	V3LHS_356183	AGGCAAGTGTTGGAGAATA
shSLUG-A		ATTTGACCTGTCTGCAAATGC
shSLUG-A		TGTGAGTTCTAATGTGTCC
shRandom		ATCTAGGACGTGGCAAGCAAC

Table M1- shRNA vector sequences

Briefly, lentiviral infection was achieved transfecting HEK293T cells with the 3rd generation packaging systems vectors pMD2.G and psPAX2, together with lentiviral vectors expressing shRNA sequences. Lentiviral supernatants were harvested 48 hours post-transfection, filtered and supplemented with protamine sulphate (4μg/ml). Supernatants were firstly titrated to measure the amount of viral particles by evaluating GFP-expressing positive population on cell lines of interest. The optimal amount of lentiviral supernatant needed for efficient transduction such as MOI (multiplicity of infection) was calculated applying the formula:

(Total number of cells seeded) x (Desired MOI) = Total transducing units needed (TU);

(Total TU needed) / (TU/ml) = Total ml of lentiviral particles to add to cells.

Cells were then infected with the correct amount of virus and recovered after 24 hours. pGIPZ infected cells were selected by using Puromicin (Gibco, Invitrogen).

miR-200c over expressing cells (231 mir200c and 157 mir200c) and control cells were obtained from MDA231 and MDA157 by stable retroviral infection with pLNCX-miR200c and pLNCX2 vectors, respectively, kindly provided by Dr. Marcus Peter, (Northwestern University, Chicago) *(Schikel, 2010)*.

For retroviral infection, LinxA packaging cell line was transfected with pLNCX vectors. Culture supernatants were collected after 48h and used to infect MDA231 and MDA157. Infected cells were selected by using G418 (Gibco, Invitrogen).

3D culture conditions and Sphere Forming Efficiency (SFE)

Single cell suspensions derived from monolayer cultures were maintained in a serum-free medium referred as *stem-medium* consisting in DMEM F-12 medium (Gibco) supplemented

with 4 ug/ml heparin (Sigma), 0,6% glucose, 0,1% NaHCO3, 100 ug/ml apotransferrin, 25 ug/ml insulin, 9 ug/ml putrescine (1-4 diaminobutane dihydrochloride) (Sigma) and 3x10-4 M sodium selenite (Sigma) in ultra low attachment plates (Corning). Every other day culture medium was enriched with fresh 20 ng/ml EGF and 10 ng/ml bFGF (Peprotech). Mammospheres were collected by centrifugation every 5-10 days and dissociated enzymatically (5 minuts in 0,05% Trypsin/EDTA) and mechanically by pipetting. Single cells obtained were re-plated for secondary and tertiary cultures, collected for RNA and protein.
For Sphere Forming Efficiency single cell suspensions derived from monolayer cultures were plated at a density of 5000 cells/well into 96-well ultra low attachment plates (Corning). Cells were maintained in the medium conditions above mentioned. Cells were allowed to form spheres up to 10 days. The number of spheres (>50 cells/sphere) per well was then counted and expressed as ratio of speres/1000 plated cells. Mammospheres were collected by centrifugation and mechanically dissociated by pipetting. Single cells obtained were counted and re-seeded for secondary and tertiary cultures.

Flow cytometry analysis

Monolayer cultured cells were detached in PBS 2mM EDTA, washed in cold PBS, counted and resuspended at $1x10^6$ cells/100 µl in Blocking Solution (PBS 10% Rabbit Serum, Dako). Cells were labelled with anti-human CD24-PE/Cy7 (BioLegend), CD24-PE (BD, Biosciences), CD44-FITC (BD, Biosciences), CD133/1-PE (Miltenyi Biotec), EpCAM-PerCP-Cy5.5 or EpCAM-APC (BD, Biosciences), CD10-PECy5 (BD, Biosciences), E-cadherin-APC (BioLegend) or isotype controls according to the suppliers' protocols. Then cell samples were washed, resuspended in cold PBS 2mM EDTA, examined on FACSCanto (BD, Biosciences) or Cytomics FC500 (Beckman Coulter) and analyzed by FACSDiva software (BD, Biosciences) or CXP Software (Beckman Coulter), respectively.

RNA extraction and qRT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen). mRNAs were reversed transcribed into cDNA with SuperScript II Reverse Transcriptase (Invitrogen) and random primers in according with the supplier's protocol. For SOX2 analysis, total RNA was reverse transcribed using a modified poly Toligo (5'GAACGAGACGACGACAGACTTTTTTTTTTTTTTTTTTTVVN).

Quantitative RT-PCR (qRT-PCR), using gene-specific primers, was carried out in triplicate with SsoFast EvaGreen Supermix (BioRad) and the CFX96 Real-Time System (Bio-Rad). Relative expression levels were normalized to controls by using the comparative Ct (ΔΔCt) method and the geometric average of a set of three housekeeping genes (GAPDH, β2-microglobulin and 18S) by the Bio-Rad CFX manager software. Specific primer pairs have been reported below (**Table M2**).

RT-PCR was performed using one microliter of the cDNA reaction in a 35 cycles of PCR amplification using Taq Polymerase kit (Promega).

Gene symbol	Sense primer	Antisense primer
B2M	GAGTATGCCTGCCGTGTG	AATCCAAATGCGGCATCT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
RN18S1	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ALDH1A1	GTTAGCTGATGCCGACTTGG	CCCACTCTCAATGAGGTCAAG
ALDH1A3	GCAACCTGGAGGTCAAGTTC	CCTCTGGAAGGCAACCTGT
E-CADHERIN	AAGGAGGCGGAGAAGAGGAC	CGTCGTTACGAGTCACTTCAGG
ESR1	TGGGCTTACTGACCAACCTG	CCTGATCATGGAGGGTCAAA
FOXA1	GGG TGG CTC CAG GAT GTT AGG	GGG TCA TGT TGC CGC TCG TAG
HEY1	GACCATCGAGGTGGAGAAGG	CGGCGCTTCTCAATTATTCC
KRT18	TGGAGCCATTACTTCAAGATCA	TGGCCAGCTCTGTCTCATAC
KRT19 *	ACTACAGCCACTACTACACGACCAT	GTCTCAAACTTGGTTCGGAAGTC
NOTCH1	GACTCAGCAGCACCTGGATG	GATCATGAGCGGGGTGAAG
SNAI2	TGCAAGATCTGCGGCAAG	TCAGAATGGGTCTGCAGATG
SOX2	TTGAATCAGTCTGCCGAGAA	GAACGAGACGACGACAGAC §
TWIST1	TCCTCTACCAGGTCCTCCA	GGAAACAATGACATCTAGGTCTC
VIM	ACAACCTGGCCGAGGACATC	AGAGACGCATTGTCAACATCCTG
ZEB1	CAGGCAGATGAAGCAGGATG	GACCACTGGCTTCTGGTGTG
ZEB2	GCGCTTGACATCACTGAAGG	ACCTGCTCCTTGGGTTAGCA

Table M2- Specific gene primer pairs used

** Zhou, 2008*

§ Primer specific for the oligo used in the reversion transcription.

For miR200c analysis, RNA was converted into cDNA and amplified in the qRT-PCR by using TaqMan specific kits for miR200c or for RNU48, as a normalizer, (Applied Biosystems). Again, relative miRNA expression was determined by the ΔΔCt method.

Statistical differences between means obtained from three experiments were evaluated using Student's t test.

Western blot analysis

Western blotting was performed on cell protein lysates (50 mM Tris, pH 7.6, 250 mM NaCl, 0,2 % Triton X-100, 0,3% NP40, 2 nM EGTA, 2 nM EDTA, 0.1% P-40, complete protease inhibitor cocktail Roche Molecular Biochemicals) using mouse antibodies reacting to human Cytokeratin-19 (Abcam), E-cadherin (BD, Biosciences), GAPDH (Chemicon International) and rabbit antibodies to human claudin-3 (Invitrogen), claudin-4 (Invitrogen), EGFR (Cell Signal), Phospho-EGF Receptor-(Tyr1068) (Cell Signal), SOX2 (Abcam), SNAI1 (Cell Signal), SNAI2 (Cell Signal), ZEB1 (Santa Cruz Biotechnology Inc.), SIP1/ZEB2 (Bethyl, Laboratories Inc.). Immunoreactivity was detected with anti-mouse and anti-rabbit secondary antibodies HRP-labeled (PerkinElmer) using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer) by the imaging analyzer Chemidoc XRS+ (Biorad). Protein expression was analyzed using the ImageLab imaging software (Bio-Rad).

MTT assay

Sensitivity to EGFR inhibitor (324674; Calbiochem) was evaluated by MTT assay. Cells were plated in triplicate at a density of 5000 cells/well into 96-well plates, incubated overnight and the following day exposed to increasing doses of EGFR inhibitor, dissolved in ethanol. A corresponding volume of ethanol was added to the untreated cells used as a control. After 48 hours, cells were labeled with reagents of MTT kit (Roche) according to the manufacturer's instructions. The absorbance at 540 nm was measured by the microplate reader Infinite 200 (TECAN). The MTT assays were performed twice. The surviving fraction (% viability) was calculated as the percentage between colorimetric value of treated and untreated cells for each dose.

SRB assay

The sulforhodamine B (SRB) (Sigma) assay was used for cell proliferation rate determination. Cells were seeded in triplicate at a density of 2000 cells. After culture, cells were fixed with

10% (wt/vol) trichloroacetic acid, washed in deionized water and stained with 0,4% (w/v) SRB diluted in 1% (vol/vol) acetic acid for 20 min, after which the excess dye was removed by washing repeatedly with 1% acetic acid. The protein-bound dye dried was dissolved in 10 mM Tris base solution for absorbance determination at 510 nm, measured by the microplate reader Infinite 200 (TECAN). The SRB assays were performed twice. The proliferation rate was determined as the increment of cells of a given day (1 to 6) in respect to cells at day 1.

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