Università degli Studi di Milano
Scuola di Dottorato in Medicina Molecolare

Curriculum di Genomica, Proteomica e tecnologie correlate
Ciclo XXV
Anno Accademico 2011/2012

Tesi di Dottorato di Ricerca:

THE ROLE OF miR-199a IN BREAST CANCER PROGRESSION AND METASTASIS

Dottoranda: Valentina MARTINO
Matricola: R08663

Direttore della Scuola: Ch.mo Prof. Mario Clerici
Tutore: Ch.mo Prof. Mario Clerici
Co-tutore: Dott.ssa Ileana Zucchi

SOMMARIO

Le metastasi rimangono la principale causa di morte in pazienti affetti da tumore. La transizione epitelio-mesenchimale (EMT) viene indicata come uno degli eventi preliminari all'instaurarsi di metastasi. L'EMT infatti, consente alle cellule epiteliali tumorali di acquisire la capacità di migrare e di colonizzare nuovi tessuti ed organi. Tale processo è un trans-differenziamento attivo durante lo sviluppo embrionale e porta alla formazione di cellule mesenchimali a partire dai foglietti epiteliali dell'embrione, provocando la perdita di giunzioni intercellulari e l'acquisizione di motilità. La comprensione dei *pathway* molecolari associati all'EMT ed alla promozione di metastasi è essenziale per lo sviluppo di nuove e più mirate strategie terapeutiche per l'eradicazione dei tumori.
Il nostro gruppo di ricerca ha sviluppato un modello di carcinogenesi mammaria basato sulla linea cellulare di ratto LA7. Le cellule appartenenti a questa linea hanno caratteristiche tipiche delle cellule tumorali staminali (CTS) e, se iniettate in animali immuno-compromessi in singola cellula, ricapitolano l'intero processo di sviluppo tumorale inclusi il processo di EMT e la formazione di metastasi. Utilizzando il sistema LA7 è stato possibile individuare le cellule fibroblastoidi generate durante la progressione tumorale, dalla progenie epiteliale della cellula LA7 iniettata (cellule LA7-Elongated). Il microRNA-199a è stato individuato come uno dei fattori up-regolati durante EMT. In questo lavoro abbiamo dimostrato che l'azione del miR è diretta contro le proteine responsabili della formazione delle giunzioni aderenti intercellulari, E-Caderina e β-Catenina. Queste ultime sono considerate *gatekeepers* del fenotipo epiteliale ed oncosoppressori. Abbiamo individuato nella regolazione post-trascrizionale della proteina tirosin-fosfatasi PTPRF, un evento scatenante il processo indotto dal miR-199a. PTPRF è anch'essa considerata onco-soppressore per via del controllo effettuato sullo stato di fosforilazione di E-Caderina e β-Catenina, indispensabile per il mantenimento delle loro affinità di legame durante la formazione dei complessi di adesione cellula-cellula. Inoltre, la stimolazione delle cellule LA7 con la citochina TGF-β, nota per le sue capacità di induzione dell'EMT, porta alla produzione del miR-199a attraverso l'induzione del fattore trascrizionale Twist1. Abbiamo inoltre dimostrato come l'inibizione dell'azione del miR-199a sui suoi target, sia sufficiente per abrogare le capacità invasive e migratorie delle cellule LA7E in matrici tridimensionali.
Infine, il trapianto in topi NOD/SCID di cellule LA7 ingegnerizzate allo scopo di modulare i livelli di espressione del miR-199a, ha portato al fallimento dell'attività metastatica di queste cellule anche se la loro capacità tumorigenica non è risultata affetta.
I risultati di questo progetto di ricerca individuano nel miR-199a un fattore responsabile del coinvolgimento delle cellule epiteliali LA7 nel processo di EMT e del loro ingresso nella cascata metastatica. Lo studio del ruolo dei microRNA, individuati grazie all'utilizzo del sistema cellulare LA7, nella progressione tumorale è in corso nel nostro gruppo di ricerca. L'acquisizione di nuove conoscenze sul processo di carcinogenesi mammaria darà un contributo indispensabile per l'individuazione di nuovi fattori prognostici e target terapeutici.

ABSTRACT

Metastasis remains one of the leading causes of death in cancer patients. The epithelial to mesenchymal transition (EMT) is proposed as a preliminary event underlying the metastatic process, allowing tumor cells to migrate and colonize new tissues and organs. EMT is a trans-differentiation program active during embryonic development that produces mesechymal-like cells from epithelial sheets by loss of cell adhesion and leading to increased cell motility. Dissecting molecular pathways associated to EMT and to its metastasis-promoting potential is essential to develop therapeutic strategies against cancer metastasis.

Our group developed a model of mammary carcinogenesis based on the rat cancer stem cell line LA7, that when engrafted at the single cell level in immuno-compromised mice, recapitulates the entire process of tumor development including the EMT process that lead to metastasis formation, through the generation of fibroblast-like cells (LA7-Elongated cells). Using the LA7 system we found miR-199a up–regulated during *in vivo* EMT transition. Ectopic expression of the miR-199a in LA7 induced cell shape modification and changes in marker expression coherent with an epithelial to mesenchymal trans-differentiation, recapitulating the fibroblast-like LA7 counterpart phenotype (LA7-Elongated). These results suggest that miR-199a is an effective inducer of EMT. We demonstrate that the action of miR-199a is directed on adherens junction proteins such as E-Cadherin and β-Catenin, gatekeepers of EMT, through leukocyte antigen-related tyrosine phosphatase (LAR or PTPRF) protein down-regulation. Furthermore, the induction of EMT in LA7 by TGF-β treatment resulted in miR-199a up-regulation through TWIST1 induction, while the down-regulation of miR-199a abrogated the invasion capacity of the LA7-Elongated fibroblast-like cells in 3D cell culture assays.

Moreover when injected in NOD/SCID mice, LA7 cells in which we induced modulation of miR-199a were unable to form metastasis, even if their tumor seeding ability was not affected.

Taken together our results support that miR-199a is a component of the pathway that commits epithelial cells to the EMT program and an important factor in the metastatic cascade engagement. Research concerning miRs identified using the LA7 model system and their possible use as therapeutic agents is ongoing in our lab and will provide new useful tools for cancer therapy.

INDEX

1.INTRODUCTION

1.1 Mammary gland structure and development

The adult mammary gland (MG) is a tree-like structure composed of hollow branches whose main function is the production and delivery of milk from mother to newborns. MG branches are constituted by a bilayered polarized epithelium (fig.1.1) which consists of:

- an inner layer of luminal epithelial cells that surround a lumen and differentiate into the alveolar structures that produce milk;
- an external layer of mioepithelial cells that secrete the basal lamina separating the mammary arbor from the stroma. The actin microfilaments of myoepithelial cells provide contractility to facilitate milk release upon lactation.

The entire epithelial ductal tree is embedded in a network of connective tissue and complex stroma constituted of adipocytes, fibroblasts, immune and endothelial cells called fat pad.

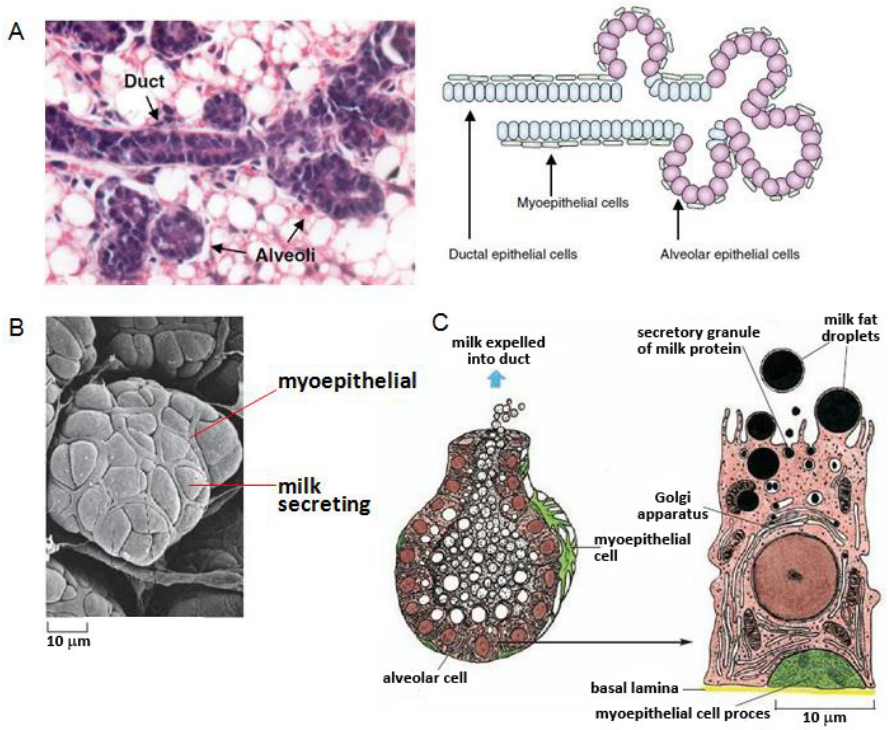


Figure 1.1. A *Hematoxylin and eosin staining of a mouse mammary duct during pregnancy and its schematic representation show the bilayered epithelium and the alveolar structures (adapted from [1]).* ***B*** *Scanning electron micrograph of a lactating rat mammary gland showing myoepithelial cells surrounding an alveolar structure (adapted from [2])* ***C*** *Schematic representation of alveoli and alveolar cells secreting milk proteins and fat (adapted from [3]).*

1.1.1 Development of the mammary tree

Development of the mammary gland is an highly complex and dynamic process in which proliferation, differentiation, apoptosis and migration events cooperate to give rise to an highly organized network of tubular branches of epithelial cells [4]. Mammary gland develops undergoing distinct stages throughout embryonic and pubertal development and reproductive life. At each stage, different signals are required to induce changes in both the epithelium and the surrounding mesenchyme [5].
The organogenesis of the mammary gland begins in embryogenesis, is independent from hormones stimulation and is based on the continuous and reciprocal crosstalk between the epithelium and the stroma [5,6]. After birth, during the time of ovarian hormone release at puberty, the branching morphogenesis that is required for the development of the ductal tree takes place. During this time the distal ends of the mammary ducts develop into structures composed of stratified epithelial cells, called the terminal end buds (TEBs) (fig.1.2).

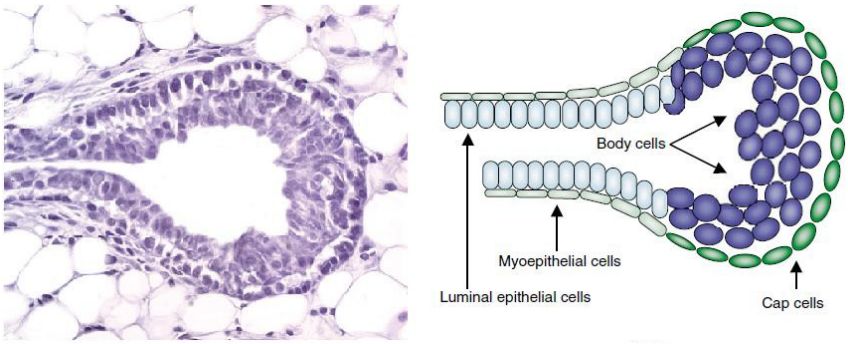


***Figure 1.2**. Hematoxilin and eosin staining of a TEB elongating in to the fat pad and its schematic representation shows a multilayered epithelium (body cells) surrounded by a layer of cap cells where stem cells are thought to reside (adapted from [1]).*

TEBs are highly proliferative structures at the invading fronts of the ducts that extend and branch into the fat pad until it is completely filled (fig.1.3). The final developmental fate of the mammary gland is accomplished during pregnancy and lactation. Upon stimulation by reproductive hormones, the mammary epithelium expands and differentiates into milk-producing lobular alveoli [4]. Eventually, the secretory epithelium undergoes apoptosis and the mammary gland is remodeled back to the starting quiescent state [8,9].

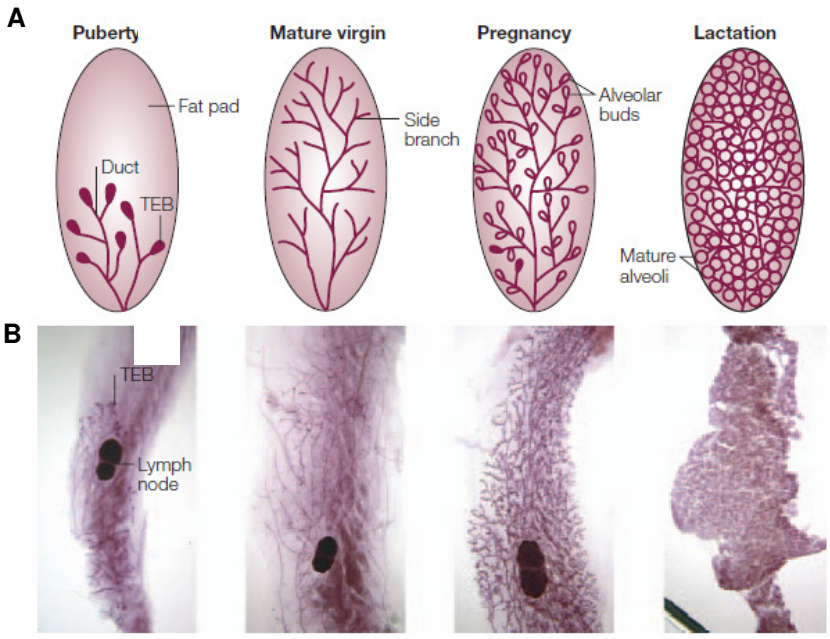


Figure 1.3. Evolution of the mammary tree. A *schematic and* ***B*** *whole mount representation of different stages of the mammary gland development from puberty to pregnancy and lactation (adapted from [5]).*

1.1.1.1 Mammary stem cells

Mammary gland is a highly plastic organ that undergoes several cycle of dramatic expansion and involution during a woman's lifetime. The task of mammary gland regeneration through cycles of pregnancy, lactation and involution is attributed to stem cells that are proposed to reside in the mammary epithelium [10].

The formation and maintenance of various tissues in the body follow a common hierarchically organized scheme. Figure 1.4 summarizes the mammary gland cell hierarchy. At the summit of the hierarchy, stem cells (SC) are a rare population of the adult tissues whose primary functions are tissue homeostasis maintenance and wound repair. Adult SC have the capacity to generate daughter cells that will differentiate into all the cell lineages that are found in the mature tissue [11]. The resident stem cell population can be maintained due to their self-renewal ability of SC that go through asymmetric division to generate an identical cell and a distinct cell that will enter the differentiation cascade [12]. Stem cell pool resides in an active environment, called niche, that is indispensable to regulate SC number, proliferation and differentiation through signaling factors release [13, 14].

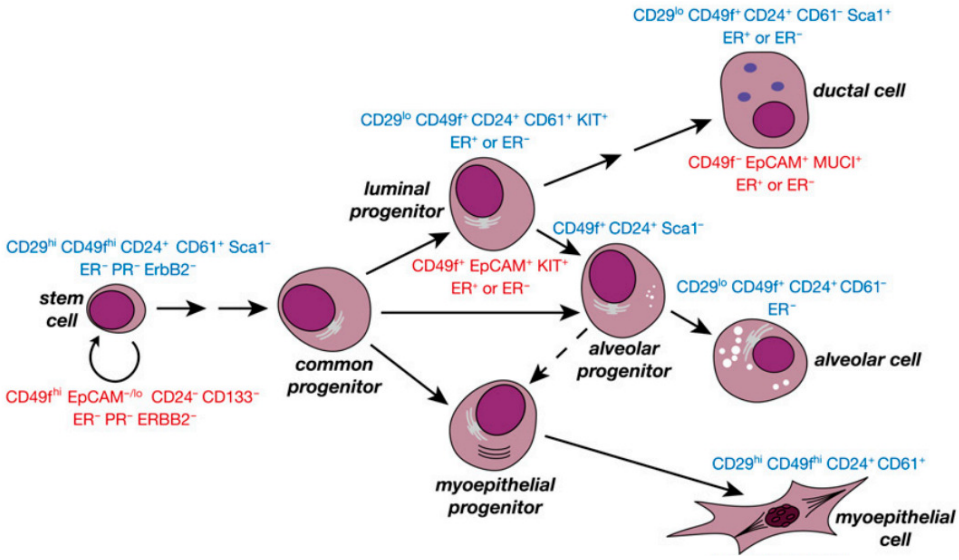


Figure 1.4. Hierarchical model of differentiation within mammary epithelium. *Primary cell surface markers used in the isolation of mouse and human epithelial cell subsets are shown in blue and red, respectively (adapted from [11]).*

Almost 60 years ago, DeOme and colleagues [15] showed that portions of the mammary gland could reconstitute the entire mammary ductal tree if transplanted into the mouse mammary fat pad that had been cleared of its endogenous epithelium. This transplantation system has provided a first *in vivo* proof of the existence of mammary SC (MaSCs). Later Smith and Medina [16] revealed that repopulating mammary cells exist in the adult mouse and further studies [17] demonstrated the existence of stem cells using retroviral marked mammary epithelial fragments. In the human breast the existence of MaSCs was implied by the presence of cells within mammary ducts that exhibit clonal derivation and by the distinctive phenotypes that are produced by primary breast epithelial cells in differentiating culture [18]. Since human MaSCs (hMaSCs) are unable to grow in the mouse fat pad environment, direct proof of hMaSC existence based on reconstitution assays after xenograft, represented a challenge for long time. Kuperwasser and colleagues demonstrated that, if supported by human fibroblasts pre-colonizing the mouse fat pad and recreating the niche, human mammary organoids or dissociated human mammary cells are able to grow *ex situ,* regenerating recognizable human mammary epithelial structures after transplantation [19, 20]. Other evidences of hMaSC existence were provided recently, when reconstitution of mammary tissue starting from cells dissociated from normal human mammary tissue was accomplished after transplantation under the mouse kidney capsule [21].

1.1.1.2 Coordinated epithelial motility during mammary gland morphogenesis

During puberty, the mammary gland develops by the branching morphogenesis of TEBs that consists in extension, bifurcation and differentiation of a rudimentary epithelial bud into a mature tubular organ [5].

TEBs are embedded in the complex environment of the fat pad (fig. 1.5), in which macrophages and eosinophils, recruited in part by signals released from the epithelium, adipocytes and mast cells coexist and create the differentiating cues that, together with mechanical stress, will ultimately give rise to the mature MG. Crosstalk between the epithelial and stromal compartments is mediated by growth factors such as insulin-like growth factor 1 (IGF1), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) families which bind to their cognate receptors to induce cell proliferation, survival and branching. WNT, Hedgehog and transforming growth factor-β (TGFβ) pathways are activated as indispensable modulators of the process. Matrix metalloproteinases (MMPs), degrading enzymes of the extracellular matrix (ECM) components display spatially localized expression and activity allowing correct patterning of MG. Tissue inhibitor of metalloproteases (TIMPs), MMPs and disintegrin and metalloproteinase domain-containing proteins (ADAMs) operate at the stromal-epithelial interface to affect bioavailability of ECM bound growth factors or regulating ligands-receptors complexing at cell surface [23]. Integrin-dependent ECM signaling and mechanical cues are also emerging as additional regulators of morphogenesis [24].

Live-cell imaging revealed that TEBs are compact multilayered bulbs of cells that invade as a cohesive group through the surrounding stroma [25, 26, 27]. This mechanism differentiates the MG evolution from other system of organ branching morphogenesis, such as salivary gland, where clear leader cells can be distinguish at the extending bud tips. Branching of the MG can thus be considered a form of collective migration, in which a population of cells uses actomyosin contractility to invade the fat pad [28, 29, 30]. Although epithelial cells remain connected to each other and membrane protrusions are not observed, non-classical epithelial traits like reduction in polarity and rapid dynamic cells reorganization are evident. These evidences prompted the suggestion that an epithelial-mesenchymal transition (EMT) or an EMT-like mechanism may be involved in the branching process[31, 32, 33]. EMT is considered one of the primary strategies adopted during development to increase epithelial cell motility. Nevertheless new evidences suggest that rather than be an all-or-nothing switch, EMT can be efficiently tuned resulting in a partial trans-differentiation that allows efficient migration of cohesive epithelia while maintaining cells connectivity and internal organization [33]. Accordingly, even in the absence of scattering or actin-rich protrusion at the invasive front [25], mammary TEBs show increased expression of the mesenchymal marker Vimentin, of the Snai and Twist families of transcription factors at the leading edge of growing branches [34]. Moreover the Snail, Slug, and E47 E-box-binding transcription factors have been proved to be key promoters of branching through repression of E-cadherin [35]. All of this are hallmarks of EMT that, as it will be mentioned later, are thought to be reactivated upon neoplastic transformations.

Homology between morphogenic migration and tumor dissemination stresses the need for a deeper comprehension of developmental mechanisms that will provide precious tools to prevent and produce effective therapies against breast malignancies.

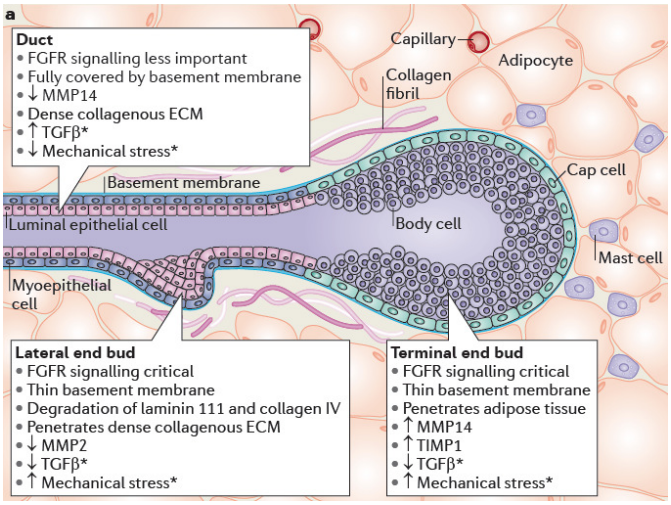


Figure 1.5. *Different cues act on different cellular comparts of the growing TEB to direct correct morphogenesis (adapted from [4]).*

1.1.2 Polarized adhesions maintain mammary gland architecture and integrity

Mammary gland is composed of a polarized epithelium whose cells show an asymmetric distribution of protein on the plasma membrane. Tight cell-cell contacts in the luminal layer participate to the establishment and the maintenance of apical-basal polarity that guarantees the functionality of the organ. Intercellular adhesions are formed by means of different protein complex: adherens junctions (AJ), tight junctions (TJ), gap junctions and desmosomes. AJ complexes are calcium-dependent, cadherin-based anchoring junctions that together with desmosomes organize and tie micro and intermediate filaments of the cytoskeleton to the plasma membrane. AJs are uniformly distributed along the plasma membrane forming a continuous belt, subjacent to the tight junctions. Trans-membrane cadherins mediate cell–cell adhesions through the homophilic binding of their extracellular domains, while the cytoplasmic tails bind to armadillo family members providing anchoring sites for the actin cytoskeleton [36].
TJs localize at the apical and basolateral domain, where they function as a barrier sealing the space between neighboring cells. The tight junction transmembrane proteins occludin, claudin and junctional adhesion molecule (JAM) bind through their cytoplasmic domain to several intracellular scaffolding proteins, including zonula occludens (ZO1-3) (fig.1.6) [37].

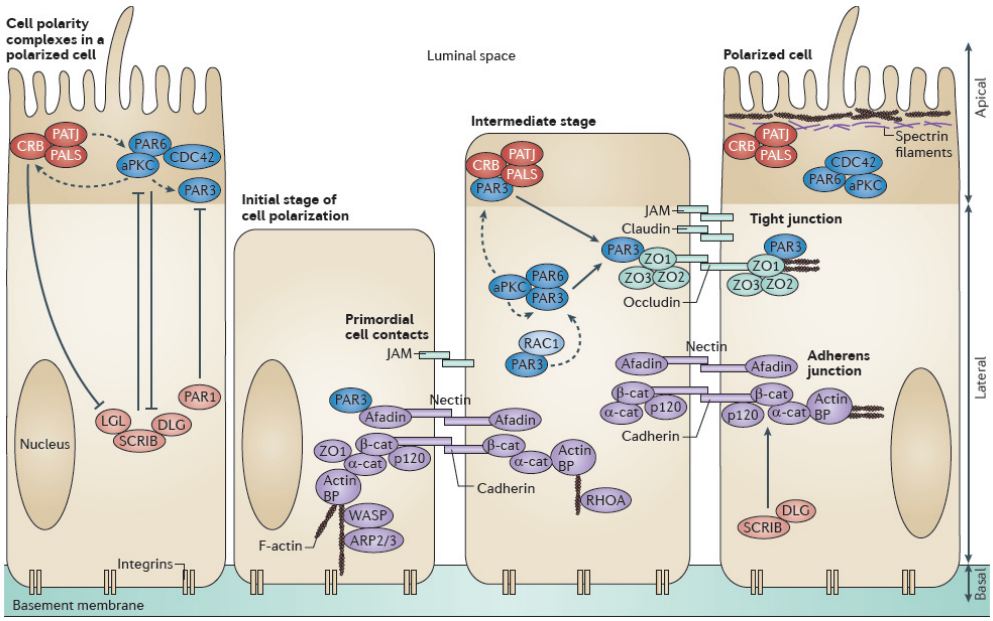


Figure 1.6*. Epithelial mammary cells are polarized. Establishment of polarity by epithelial cells depends on sequential steps of adhesion formation and polarity proteins correct localization. Final asymmetric distribution of adhesive proteins that guarantee functionality of secreting epithelium is shown [38].*

Polarity allows cells to sense and to organize the proper spatiotemporal responses to stimuli that arise from adjacent cells and the surrounding microenvironment. In addition, polarity allows directional vectorial transport typical of a secreting epithelium cells [39].

The spatial asymmetric localization of junctional complexes is mediated by an evolutionarily conserved class of proteins that belongs to the Pulmonary adenoma resistance (Par), the Scribble and Crumbs (CRB) families. Complexes formed by members of these families contribute to define the membrane topology [39]:

- PAR complex: comprises PAR3, PAR6, atypical protein kinase C (aPKC) and cell division control protein 42 (CDC42) and promotes the establishment of the apical-basal membrane border;
- CRB complex: is formed by the transmembrane protein CRB and the associated cytoplasmic proteins PALS1 and PALS1-associated tight junction protein (PATJ), and is required to establish the apical membrane;
- Scribble complex: is formed by scribble homologue (SCRIB), lethal (2) giant larvae homologue (LGL or LLGL) and discs-large (Drosophila) homologue (DLG), and defines the basolateral plasma domain.

Loss of expression or functional activity of cell polarity and cell adhesion proteins is related to developmental bias and to tumor progression and invasiveness. Table 1.1 summarizes known alteration in polarity protein expression linked to human neoplasias. Polarity pathways result to be deregulated under activation of oncogenes such as v-K-ras, RhoA, Rac1, Raf-1 or during EMT-like processes [40,

41, 42]. Upon polarity disruption, cells may become unresponsive to growth inhibitory signals thus avoiding differentiation, senescence or apoptosis. Loss of polarity complexes is also associated with alterations in epithelial functions and mitotic defects that may lead to the generation of genomic instability [43]. Furthermore, down-regulation of the polarity proteins can directly deregulate cell adhesion processes, which in turn will disrupt morphogenesis and promote tumorigenesis [44]. The deregulation of AJs by mutation, methylation or transcriptional repression of E-cadherin has been proposed as preliminary event in solid cancer progression [45]. All this evidences prompted the new concept that polarity and adhesive proteins are tumor suppressors. Intercellular adhesions and polarity factors have essential roles in the establishment and maintenance of epithelial tissue organization and integrity. Understanding their regulatory networks is essential to provide novel insights into the mechanisms by which cells and tissues structure is lost in tumors of epithelial derivation (carcinoma).

Gene (protein)	Alterations	Cancer type	Phenotypes
Crumbs complex			
CRB3 (crumbs 3)	Downregulated expression	Human tumour epithelial-derived cell lines	Cell–cell junctions disrupted and increased metastasis
PAR complex			
PARD3 (PAR3)	Gene deleted or downregulated expression	Oesophageal squamous cell carcinoma cell lines and primary tumour tissue	Cell–cell junctions disrupted
PARD6A (PAR6α)	Overexpressed	ER-positive breast cancer cell lines and primary tumour tissue	Hyperproliferation
	Overexpressed and phosphorylated	Human BRCA1-defective tumour tissues	Lumen filling, cell–cell junctions disrupted and increased metastasis
	Overexpressed	Stromal cells in non-small-cell lung cancer tissue samples	Associated with good prognosis
PRKCZ (PKCζ or aPKC)	Overexpressed	Human hepatocellular carcinoma samples	Hyperproliferation
	Overexpressed	Bladder tumour cell lines and primary tumour tissues	Correlated with invasiveness
	Overexpressed and phosphorylated	Dysplastic oral epithelial tissue samples, squamous cell carcinoma of the head and neck tissue samples and cell lines	Increased cell proliferation
	Overexpressed	Pancreatic cancer tissues	Invasive and metastatic phenotype
	Overexpressed	Samples of hyperplastic enlarged lobular units of precancerous breast lesion	Increased cell proliferation
PRKCI (PKCι or aPKC)	Gene amplified and protein overexpressed and mislocalized	Ovarian cancer tissue samples	Associated with low survival rate
	Overexpressed and phosphorylated	Hepatocellular carcinoma tissue samples	Associated with metastasis and invasion
	Overexpressed	Non-small-cell lung cancer cell lines and primary tumour tissues	Associated with poor prognosis
	Overexpressed	Primary breast cancer tissue samples	Associated with larger tumours, invasion and metastasis
	Overexpressed	Pancreatic cancer tissue samples	Tumour angiogenesis and metastasis
SCRIB complex			
SCRIB (scribble)	Mislocalized or downregulated	High-grade HPV-positive cervical squamous intraepithelial lesions and invasive cervical carcinoma samples	Correlates with invasiveness
	Mislocalized or downregulated	Neoplastic colon mucosa	Loss of tissue architecture
	Mislocalized or downregulated	Human breast cancer tissue samples	Loss of three-dimensional cell polarity and inhibition of apoptosis
DLG1 (DLG)	Mislocalized or downregulated	High-grade uterine cervical neoplasm samples	Role in cytokinesis, viral trafficking and metastasis pathways
	Mislocalized or downregulated	Neoplastic colon mucosa samples	Loss of tissue architecture
LLGL1 (LGL1)	Downregulated	Tissue samples from breast, prostate, lung and ovarian tumours	Disruption of cell polarity and tissue architecture, uncontrolled proliferation and growth of neoplastic lesions
	Downregulated	Colon cancer cell lines and colon cancer primary tissue samples	Associated with advanced stage and lymph node metastases
	Aberrantly spliced mRNA and the expression of truncated protein	Hepatocellular carcinoma cell line and primary tissues	Associated with poor differentiation and large tumour size
	Mislocalized or downregulated	Samples of human gastric epithelial dysplasia and adenocarcinoma	Disruption of tissue morphology

Table 1.1. Alteration of polarity complex proteins in epithelial transformation and human cancer [38].

1.2 Breast cancer

Breast cancer (BC) is the second most common cancer worldwide after lung tumor, the fifth most common cause of cancer death, and the leading cause of cancer death in women of western countries [48].

Breast tumor is a complex disease that include a cohort of distinct subtypes with different molecular profile and clinical outcome. Gene expression profiling of large sets of tumors coupled with classic stratification based on estrogen (ER), progesterone (PR) and human EGF (HER2) receptor expression, identify six principal molecular subtypes of breast cancer [49, 50, 51]: luminal A, luminal B, $HER2^+$, basal-like, claudin-low and normal-breast like. The luminal A and B subtypes are generally associated with a good prognosis, while the HER2 over-expressing tumors, although displaying luminal features are associated with poor overall survival. The basal-like subtype is very heterogeneous, comprises 15-20% of breast cancers, it is considered among the most clinically aggressive tumors and exhibit a triple-negative phenotype (*i.e.* ER^-, PR^- and $HER2^-$). Moreover, poorly differentiated basal-like tumors have been reported to have embryonic stem cell signature [52, 53, 54]. Claudin-low subtype has recently been identified and is characterized by low expression of claudin genes and cell–cell junction proteins, including E-cadherin. The claudin-low tumors belong to the triple negative class of BC, are rich in cells that have stem features and their proteome is enriched in EMT markers [55].

Tumor development starts with a single cell that undergoes transforming events of genetic and epigenetic nature. Tumor progression is driven by the accumulation of additional genetic changes combined with clonal expansion and selection. Like for normal development, crosstalk between the epithelial and the mesenchymal components of the organ play an important role in breast tumor progression [56]. *In vivo* and *in vitro* studies demonstrated that cells belonging to the microenvironment and ECM molecules could modulate tissue specific differentiation of normal breast cells as well as growth, survival, polarity and invasive behaviour of breast cancer cells [7].

The progression of BC is defined by specific pathological and clinical stages, from ductal hyperproliferation, to ductal *in situ* and invasive carcinoma, and finally to metastatic disease, as shown schematically in figure 1.7. While during *in situ* stage the basal lamina is present and myoepithelial cells still visible, invasive BC have lost the physiological architecture of the mammary gland including the myoepithelial layer and the basal lamina. This stage will eventually progress to metastatic spread [56].

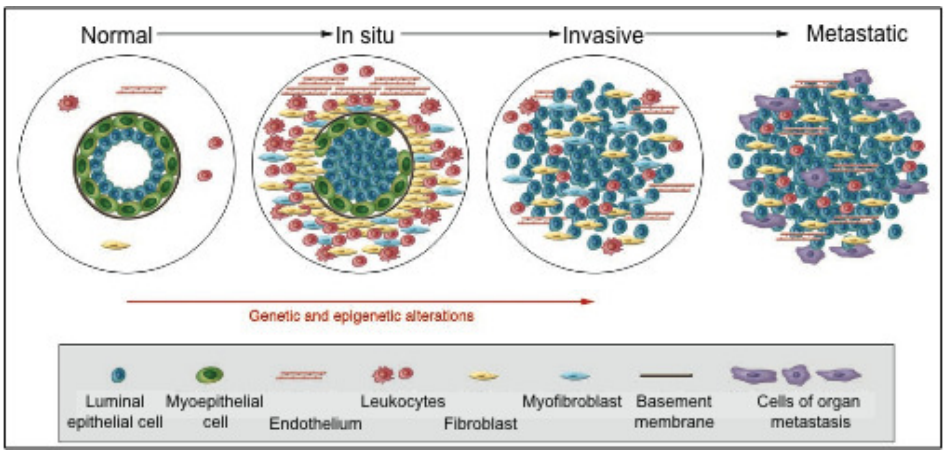


Figure 1.7. Schematic representation of breast cancer progression *(adapted from [56]).*

Despite significant advances in diagnosing and treatment of this disease, several unresolved clinical and scientific problems remain regarding prevention and early diagnosis, treatment and therapeutic resistance, tumor progression and recurrence.

1.2.1 The cancer stem cells theory

BC is a very heterogeneous disease. The heterogeneity can be observed both among tumors in different individuals (inter-tumor heterogeneity) and among different subpopulations of cells within the tumor (intra-tumor heterogeneity). The molecular mechanisms underlying inter-tumor heterogeneity are poorly defined and the tumor subtype-specific cellular origin or transforming events are unknown. The intra-tumor heterogeneity can be explained by three potential mechanisms not exclusive of one of each other:

- differences in cellular phenotypes;
- genetic diversity due to genomic instability combined with clonal selection;
- epigenetic diversity and plasticity [57].

Currently two models have been proposed to explain tumor heterogeneity, progression and therapeutic resistance: the clonal evolution and the cancer stem cell models (fig. 1.8) [58].
In both models, tumors originate from a single cell that acquires multiple mutations and unlimited proliferative potential. In the cancer stem cell (CSC) model tumors originate from a normal stem that have lost the proliferative control, or from progenitor or differentiated cell that have re-acquired stem cell properties, while in the clonal evolution model every cell can stochastically be a tumor-initiating cell. The clonal evolution model posits that genetic and epigenetic changes occur over time in individual cancer cells, conferring selective advantage to individual clones that will acquire indefinite, uncontrolled proliferation potential.

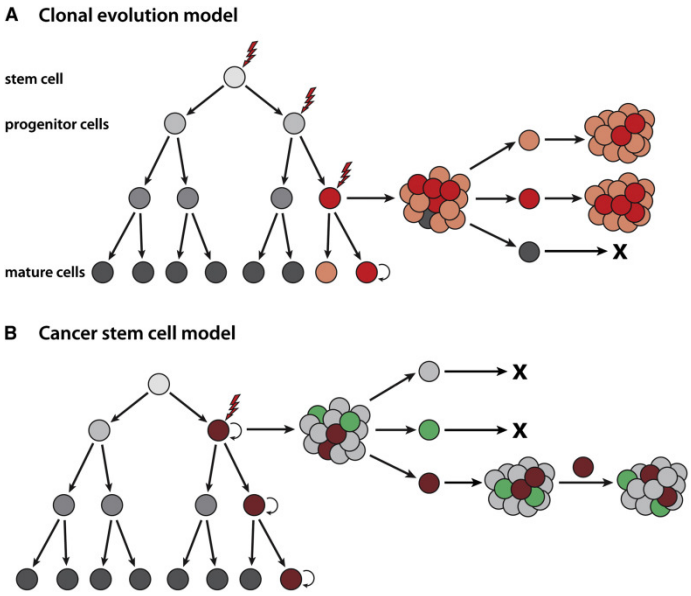


Figure 1.8. Schematic of clonal evolution and CSC models *[59].*

The CSC model was first proposed starting from evidences acquired in the hematopoietic field, in which both normal stem cells and cancer cell were well characterized [60]. This model implies that cancers are clonal and hierarchically organized similarly to the normal tissue of origin, thus only a small subset of cells is responsible for sustaining tumor growth and establishing the cellular heterogeneity of the primary tumor. Like normal SCs can undergo asymmetric division, it is proposed that CSCs can generate both a progeny of non-tumorigenic cells that will compose the bulk of the tumor mass and will eventually differentiate, and a self-renewing rare population of cells with tumorigenic potential. The evidence that many cancers often arise from and progress through the deregulation of self-renewal pathways active in normal stem cells is supporting this model.
A growing body of evidence supports the existence of CSCs in solid tumors, including malignant germ cell, brain, colon and breast cancers [61, 62, 63]. Since CSCs represent a rare population of cells, it is believed that they are maintained in a relatively quiescent state of cell proliferation. The existence of quiescent or slow-cycling cancer stem cells in tumors has implications in cancer therapy since the current anticancer therapies predominantly target highly proliferative cells, thus leaving low proliferating cells such as CSCs unaffected [59]. Since CSCs may not be eradicated, tumor formation may reoccur even years after cancer therapy discontinuation. Additionally, it has been proposed that resistance to chemotherapeutic agents may be due to the SCs and CSCs specific presence of the ABC transporters that provide multidrug resistance or to the over-expression of anti-apoptotic proteins.

1.2.2 Breast cancer metastasis

Implementation of screening and prevention programs and novel treatment strategies is decreasing breast cancer mortality [64]. However, more than 120000 deaths due to breast cancer are expected annually in the USA and Europe combined [64, 65]. Metastases will account for nearly 90% of this death. Approximately 10–15% of BC patients have an aggressive disease and develop distant metastases within 3 years after primary tumor detection. However, the manifestation of metastases at distant sites 10 years or more after the initial diagnosis is also not unusual. Moreover, approximately one-third of women who are sentinel lymph node negative at the time of surgical resection of the primary breast tumor will subsequently develop clinically detectable secondary tumors [65]. Patients with breast cancer are therefore at risk of experiencing metastasis for their entire lifetime. Improving our understanding of the molecular mechanisms of the metastatic process will improve clinical management of the disease.
Once disseminated, the common sites for BC metastatic spread are bones, lung and liver but many organs can be affected (fig.1.9).

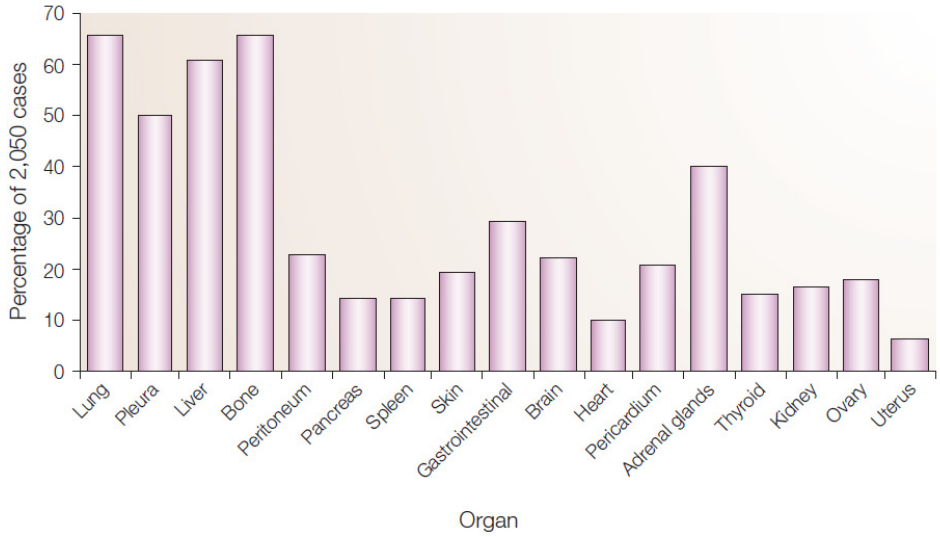


Figure 1.9 *Representation of organ specific metastatic colonization in a population of 2050 breast cancer patients [66].*

Tumor progression towards metastasis is a complex process of sequential steps that has been defined the metastatic cascade [67] (fig. 1.10). Cells must separate from the primary tumor, invade through basement membranes and surrounding tissues, enter and survive in the circulation, lymphatics or peritoneal space and arrest in a distant target organ. After extravasation, survival in the foreign microenvironment, proliferation and induction of angiogenesis are needed.
Metastatic dissemination is indeed an highly inefficient process [68]. Every step represent a threat for the survival of disseminating cancer cells that need to resist

stressful environments and to evade apoptotic death or immunological host defense mechanisms.

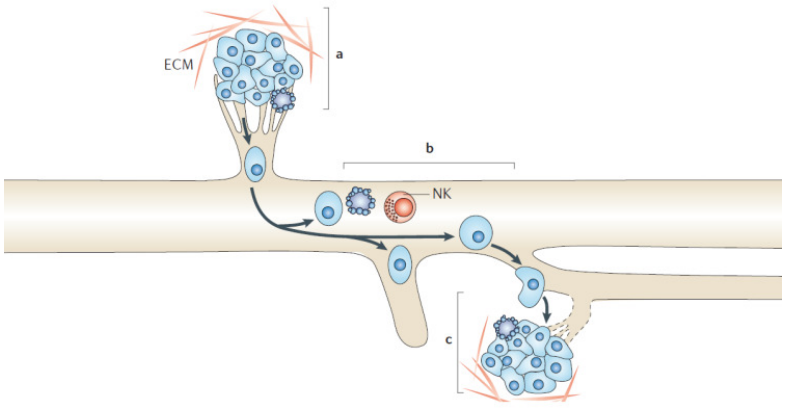


Figure 1.10. *Schematic of the metastatic dissemination.* ***a)*** *primary tumor* ***b)*** *blood stream* ***c)*** *secondary site of colonization (NK: natural killer cells) (adapted from [68]).*

1.2.2.1 Starting the metastatic cascade: invasion and migration

The initial steps of local invasion include the activation of signaling pathways that control cytoskeletal dynamics in tumor cells and the turnover of cell-matrix and cell-cell junctions, followed by active tumor cell migration into the adjacent tissue [69, 70 ,71].

Invasion is a cyclic process in which the invading cell changes shape, produces morphological asymmetry and then translocates the cell body. Depending on the cell type and tissue environment, cells can migrate in two major ways: individually, when cell-cell junctions are absent, or collectively as multicellular groups, when cell-cell adhesions are retained. Cancer cells move following the same physiological mechanisms of migration used by normal cells, but the lack of physiological stop signals immobilizing and anchoring the cells perpetuates their migration [72]. Invasive single-cell migration results from five interdependent molecular steps:

- front back polarity is established due to actin polymerization at the front of migrating cells;
- the leading edge forms transient adhesions with component of the ECM thus allowing traction force production;
- cell surface protease start degradation of ECM;
- GTPase Rho activates actomyosin contractility;
- contraction is followed by release of transient adhesion at the trailing edge that move forward while the leading edge protrude further.

When multiple cells originate from the same location such as a tumor, a leader cell forms a microtrack of locally removed ECM barriers that following cells use as a

preferential way of invasion, widening it further by mechanical force and proteolysis [73, 74].
In collective migration, cells in the invading mass behave like a single giant cell taking advantage of coordinated cytoskeletal protrusion and contractility that are mechanically mediated through cell-cell junctions [75, 76].
If monitored in time, however, invasion processes are continuously evolving from stringently collective, through partial to complete individualization, rather than follow discrete states [77]. The related concepts of EMT and mesenchymal–epithelial transition (MET), as well as partial EMT/MET in cancer, account for the heterogeneity of mechanisms of invasion.
The transition of cells from a fixed, tissue-anchored state to a mobile state is often induced by extracellular chemokines, cytokines and growth factors released by tumor cells themselves or activated stromal cells. Tumor progression is indeed intimately linked to the activation of tumor stroma which include fibroblast, endothelial cells and macrophages. After activation by tumor, this cells modify their environment in turn stimulating cancer cells and leading to the acquisition of invasiveness that starts the metastatic cascade. Migrating tumor cells acquire the ability to drive directional motility along a gradient of cell adhesion sites (*i.e* haptotaxis) or substrate-bound chemoattractants (*i.e.* chemotaxis) sensing the molecular environment through adhesion and chemotactic receptors.
The chemokine (C-X-C motif) ligand 12 (CXCL12) and EGF form stable gradients that guide migrating tumor cells and passenger leukocytes [78,79,80]. TGFβ, a known inducer of mesechymal migration, is immobilized to the ECM via fibronectin and fibrillin and is released through limited proteolysis mediated by MMPs or furin that are activated and released by activated stromal cells [80]. Osteopontin is a secreted cytokine-like proteoglycan up-regulated in many tumor types that binds to CD44 and integrins supporting invasive cell guidance [81].
Invasion patterns seen in hystopathological tumor sections, recall an aberrant reactivation of branching morphogenesis or wound healing migration mechanisms. Furthermore, the acquisition of different roles by migrating tumor cells (*i.e*. leader and follower) is reminiscent of the stable or temporary division of tasks, physiologically present among cells in the developing organ, that is determined by genetic or epigenetic modifications.

1.2.2.2 Epithelial to mesenchymal transition

EMT is a physiological trans-differentiation program activated during embryonic development and wound healing [82]. EMT enables epithelial cells to acquire phenotypic features of the mesenchymal cell thus losing their typical apico-basal polarity and strong cell-cell and cell-ECM interactions, gaining spindle like morphology and invasion/migration ability coupled with ECM degradation capacity [83]. Several distinct molecular processes are needed to accomplish EMT: activation of specific transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes and modulation of miRNAs. Figure 1.11 summarize some of the known factors involved in the transition.
Due to the overlap between skills acquired through this process and abilities required for cancer cells escape and systemic spread into patient's body, it is now

commonly accepted that metastasis cascade is dependent on aberrant reactivation of developmental EMT [84]. Indeed invading cancer cells seem to reactivate embryonic pathways and patterns of cell movement and many morphogenic signaling pathways such as Wnt, FGF and bone morphogenetic protein (BMP) signaling are relevant in cancer progression [84].

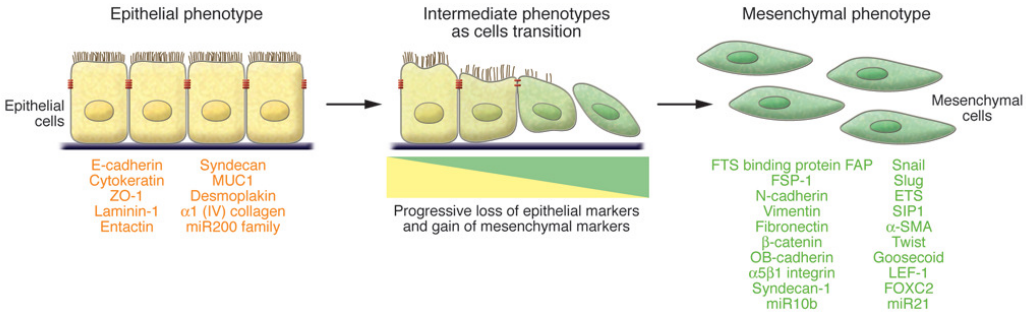


Figure 1.11 Scheme of EMT induced switch of key molecules. *Epithelial cells gradually lose their adhesive proteins and markers expression while acquiring mesenchymal proteome and miRnome [84].*

Hallmark of EMT is the loss of E-cadherin at adherens junctions that account for loss of cell polarity and disruption of tissue architecture. EMT-inducing signals such as HGF, EGF, PDGF and TGF-β originate from the inflammatory environment of the tumor-associated stroma [85]. These signals lead to the induction or activation of a series of transcription factors that can be divided into two groups depending on their ability to directly repress the gene coding for E-Cadherin (CDH1) binding its promoter. Snail, Zeb, E47, and KLF8 are direct repressor, whereas Goosecoid, E2.2, and FoxC2 act indirectly [86, 87]. There are evidences that another transcription factor, Twist, can act either indirectly through Snail2 or directly when complexed with the polycomb-group protein BMI-1 [88, 89]. As mentioned before, loss of CDH1 has been linked to tumor progression and poor prognosis. Moreover, since E-cadherin contend β-catenin to the WNT/β-catenin signaling pathway, release of free β-catenin from membrane complex due to AJs dissociation can lead to its nuclear accumulation, a typical trait of aggressive tumor. Wnt signaling activation is also critically involved in regulating the turnover and activity of Snail and Twist in the context of breast cancer [90].
Polarity loss associated with EMT is a direct consequence of EMT activator expression such as Snail and Zeb, that directly repress the transcription of polarity genes including Crumbs [91, 92]. In turn Crumbs repression enhances signaling by TGF-β, a potent activator of the EMT resulting in a positive, auto-inducible regulatory loop.
Many *in vivo* studies demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α-smooth muscle actin (αSMA), fibroblast-specific protein-1 (FSP1), vimentin and desmin. These cells localize at the invasive front of primary tumors and are considered to be the candidate cells to enter the metastasic cascade [93].

The activation of the EMT program has also been associated with the acquisition of stem cell traits by neoplastic cells. Mani and colleagues reported the induction of EMT in immortalized human mammary epithelial cells (HMLERs), by ectopic expression of either the Twist or Snail transcription factors with a concomitant expression of mesenchymal and CSC markers [94]. Another study showed that activation of Slug and SOX9 transcription factors, is able to induce SC phenotype in primary mammary cells thus increasing the mammary gland regeneration efficiency after xenograft in pre-cleared mouse fat pad [95]. These data suggest the idea that migratory cancer cell can combine stem cell and the mesenchymal cell properties necessary to efficiently complete the metastatization [96]. Moreover reprogramming of fibroblasts into induced pluripotent stem cells requires the loss of their mesenchymal character through a MET [97], compatible with the intermediate phenotype of stem cells.

1.3 MicroRNAs in cancer

MicroRNAs (miRNAs or miRs) are small (20-23 nucleotides), endogenous, non-coding RNAs highly conserved among species that participate to the post-transcriptional regulation of gene expression [98]. MiRNAs tune the expression of specific set of target mRNAs playing important roles in many physiological process like embryo development. Experimental knockdown of key enzymes of the miR maturation pathway such as Dicer, showed that loss of miRs biosynthesis is lethal [99]. Many evidences highlighted the concept that the multigene regulatory capacity of miRNAs is dysregulated in cancer: miRNA loci are targeted by genetic (about 50% of annotated human miRNAs are located either at fragile sites or at amplified/deleted regions in human cancers [100]) and epigenetic defects, showing a causal link to neoplastic development [101]. In addition, miRNA expression profiles are correlated with tumor stage, progression and prognosis in cancer patients [102, 103, 104]. Hence, specific miRNAs have been identified as regulators of cancer-related processes such as cell growth and tissue differentiation and between them many promoters or suppressors of local invasiveness of breast cancer cells have been identified. miR-10b was the first miRNA identified as regulator of BC metastasis via indirect up-regulation of RhoC activity [105]. Similarly, miR-373/520c enhances breast cancer cell invasiveness through the signal transduction molecule CD44 pathways. Induction of miR-31, which inhibits local invasiveness through the co-suppression of integrin-α5 (ITGA5), radixin (RDX) and RhoA in already established metastasis was shown to induce regression of the metastatic mass [106, 107]. Members of the miR-200 family have been shown to play an important role as gatekeepers of the epithelial phenotype by targeting Zeb, thereby preventing E-cadherin down-regulation and the EMT [108, 109]. MiR expression analysis might help the diagnosis as progtnostic factors and, due to their drugability, the treatment of cancer, therefore representing powerful tools in the definition of patients treatment strategies.

1.3.1 MicroRNA biogenesis

The biogenesis of miRNAs is well characterized and canonical pathway of microRNA maturation is shown in Figure 1.12.
miRNAs can be encoded from independent transcription units (miRNA genes) under the control of their own promoters, or can be located into the intronic regions of protein-coding genes with their expression being dependent from the host gene transcription.

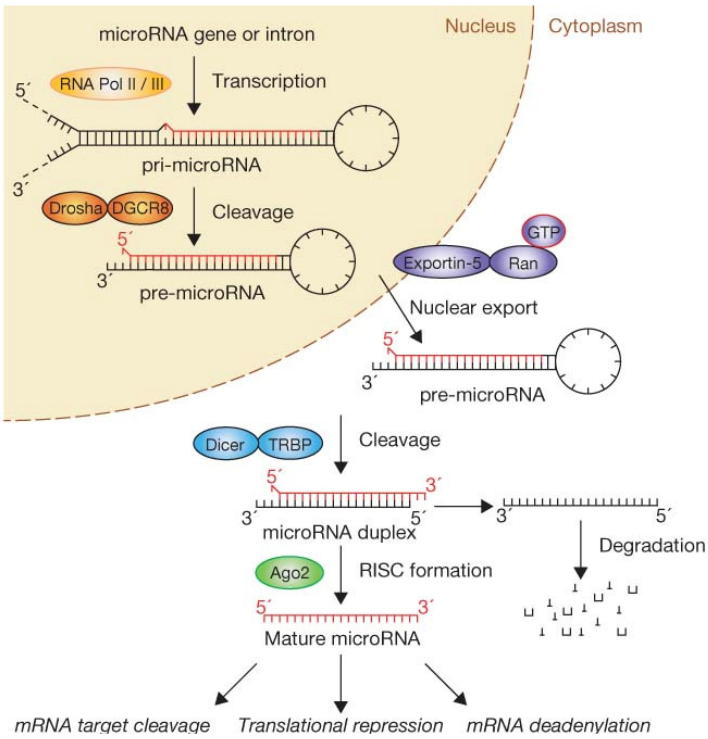


Figure 1.12. Canonical pathway of microRNAs maturation [110]

MiRNA genes are transcribed by either RNA polymerase II or III into long primary miRNA transcripts (pri-miRNA). Like other products of polymerase II transcription, many pri-miRNAs are polyadenylated and capped.
In the nucleus, pri-miRNA undergoes cleavage by the Drosha RNase III endonuclease-DGCR8 microprocessor complex generating a 60-70 nucleotide stem-loop intermediate called the miRNA precursor (pre-miRNA) [111].
The pre-miRNA is actively translocated to the cytoplasm by exportin5-RNA-GTP where it is processed by the Dicer RNase to generate a short imperfect-paired RNA duplex [112].
The functional strand of miRNA is then loaded together with Argonaute (Ago2) proteins onto the ribonucleoprotein complex RISC (RNA-Induced silencing complex) that allows its specific binding to the target sequence at the 3'

untranslated region (3'-UTR) of mRNAs. After 3'UTR pairing, the block of mRNA expression is achieved either by triggering degradation or inhibiting translation or through deadenylation [113, 114, 115, 116].
In principle, the miRNA duplex could give rise to two different mature miRNAs. However, only one strand is usually incorporated into RISC and guides the complex to target mRNAs while the other strand (passenger strand or miRNA*) is degraded. This functional asymmetry depends on the thermodynamic stability of the base pairs at the two ends of the duplex: the miRNA strand with the less stable base pair at its 5´ end in the duplex is loaded into RISC [110, 117].

1.4 A rat breast cancer stem cell model: the LA7 cell line

LA7 cells were isolated by Dr Renato Dulbecco from the RAMA-25 cell line, derived from a mammary adenocarcinoma induced in rat using DMBA (7,12-dimethylbenz[α]anthracene) [118]. Our group demonstrated that the LA7 cell line is a reliable model of mammary cancer stem cells and a suitable system to study the mammary gland differentiation.
LA7 stem cell properties can be summarize as following:
- indefinite self-renewal: LA7 are able to form mammospheres that can be regenerated for at least 80 passages *in vitro* (fig. 1.13 A)*;*
- multi-lineage differentiation potential: if exposed to lactogenic hormones or differentiating agents (*e.g.* DMSO) LA7 cells form dome-shaped structures representing the alveolar structures that develop in the mammary gland at pregnancy [119]. Moreover LA7 cells can form tri-dimensional structures *ex situ* and *in vitro* that recapitulate the normal breast architecture of tubular-alveolar structures, generating all the cell lineages of the mammary gland (fig. 1.13 B);
- tumor seeding ability: when single LA7 cells are injected into NOD/SCID mice, heterogeneous tumors are generated (fig. 1.13 C) and cells with the same features of naive LA7 cells can be re-isolated from the primary tumors [120, 121].
The mass of a single cell LA7-generated tumor is composed of different populations of cells. From the tumor bulks, three distinct cell types were individuated and isolated on the base of their distinguishable morphology and marker expression: polygonal epithelial, elongated fibroblast-like and mesenchyme-like cells. These populations resulted to have different features in terms of self-renewing and tumor seeding abilities. While cells with the same properties of naive LA7 are enriched in the population of cells with polygonal morphology, mesenchymal-like resulted to have limited life-span *in vitro.* Elongated cells (LA7E) lack mammary gland lineage specific marker expression and have a mesenchymal signature [121]. Starting to this evidences our group hypothesized that LA7E are produced by the epithelial progeny of the injected LA7 through an EMT induced during tumor development. Understanding how this population is generated and what is its role in the evolution of tumors, will give important knowledge about the metastatic progression and is the object of my thesis.

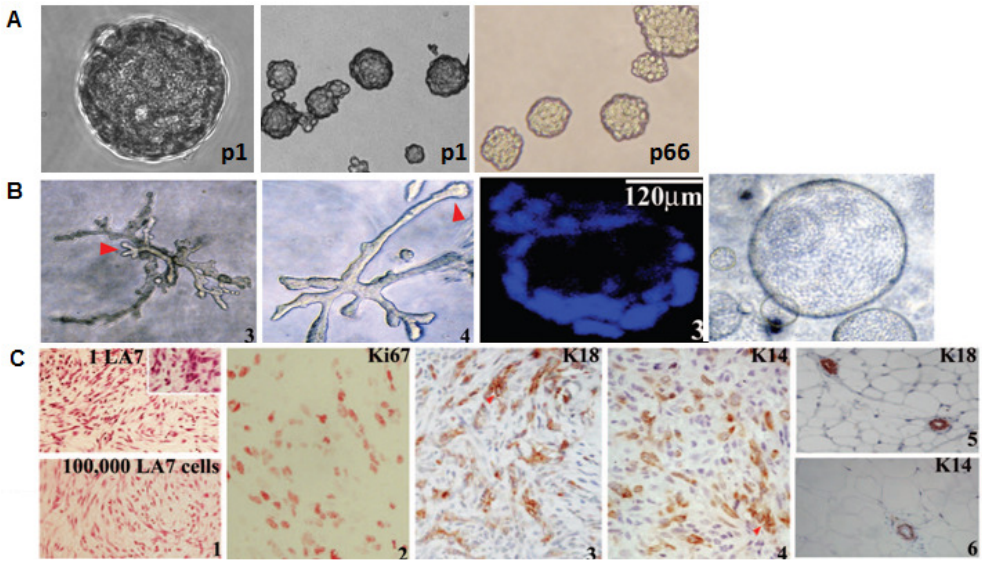


Figure 1.13 . LA7 cells have stem cells properties. A *LA7 sphere forming ability is maintained for up to 80 passages.* ***B*** *in differentiating 3D matrices LA7 recapitulates the tubule-alveolar structure of the mature mammary gland* ***C*** *LA7 generated tumors are heterogeneous and shows differentiation ability (p1: first spheres generation; p66: 66th spheres generation; Ki67: marker of cell proliferation; K18: cytokeratin 18; K14: cytokeratin 14. Adapted from [120])*

2. MATERIALS AND METHODS

2.1 Cell lines and culture conditions

Rat epithelial mammary cells LA7 and LA7E were grown in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen, Gibco Laboratories) supplemented with 10% fetal bovine serum (FBS) and 50ng/ml of both insulin and hydrocortisone (Sigma-Aldrich). Cells were grown in 5% CO_2 at 37°C. Cells are maintained in non differentiating pre-confluent condition by splitting them three times a week. For differentiation experiments in adherent cultures, LA7 confluent cells were exposed to 2% DMSO (Di-Methyl Sulfoxide; Sigma-Aldrich) and collected between 24-48 hours after treatment.
Mouse epithelial mammary cells NMU-MG were grown in DMEM (Invitrogen, Gibco Laboratories) supplemented with 10% FBS and 1µg/ml insulin (Sigma-Aldrich). Cells were seeded at 6000 cell/cm^2 and grown in 5% CO_2 at 37°C.
TGF-β (Sigma-Aldrich) was supplemented to the culture medium at the concentration of 10ng/ml. Cell culture medium was replaced every three days.
Human epithelial mammary cells MCF7 were grown in low-adhesion plates in RPMI-1640 medium (Invitrogen, Gibco Laboratories) supplemented with 10% FBS and 1µg/ml insulin (Sigma-Aldrich). Acini formation assay was performed in RPMI-1640 (Invitrogen, Gibco Laboratories) supplemented with 5% Serum Replacement (Invitrogen, Gibco Laboratories), 25ng/ml bFGF, 20ng/ml EGF (Sigma-Aldrich), 0,5ug/ml insulin (Sigma-Aldrich), 1X B27 (Invitrogen, Gibco Laboratories) and 4ng/ml heparin (Sigma-Aldrich). Cells were seeded at 1000 cell/ml.
MDA-MB-231 human epithelial cells and HEK-293, human embryonic kidney cells, were grown in DMEM (Invitrogen, Gibco Laboratories) supplemented with 10% FBS.

2.1.1 Tri-dimensional invasion assay in rat tail collagen

Rat-tail collagen was extracted as already described [122]. Monolayer cultures were detached by trypsinization and cells were resuspended in 500µl of rat-tail collagen as described at the concentration of 5000 cells/ml. After solidification, the collagen was covered with DMEM medium supplemented with 10% FBS and 50ng/ml of both insulin and hydrocortisone.

2.1.2 Cell xenografts in NOD-SCID Mice

NOD/SCID mice were injected as described [120]. Animal treatment, protocols and experiments were approved and performed under the guidelines set by the Italian Institute for Animal Care and Welfare. 1000 LA7 cells were orthotopically injected into 6 fat pads (3 mice) for each condition. Tumors were collected at different time points after injection. The size of the tumors was measured through the animal's skin; initial measurements were performed immediately after injection and continued every other day until the time of animal sacrifice. Tumors were dissected, fixed in formalin and embedded in paraffin for histological analysis.

2.2 Primary cells and culture conditions

2.2.1 Human mammary gland tumor samples

Human tissues were collected and mechanically and enzymatically dissociated. Tissues were cut in small pieces with surgical scissors and incubated in DMEM:F12 1:1 medium (GIBCO Laboratories, Carlsbad, CA, USA) supplemented with 10% serum replacement, 300U/ml Collagenase (Sigma-Aldrich) and 100U/ml Hyaluronidase (Sigma-Aldrich) for 4 hours in the incubator at 37°C, 5% CO_2. After enzymatic incubation, samples were collected in a 50ml tube, centrifuged at 0.2xg for 10 min and washed once with DMEM. Mammary gland tumors obtained from Azienda Ospedaliera Treviglio-Caravaggio, were dissociated and derived cells were maintained in a non-differentiating culture medium composed by DMEM:F12 1:1, 10% serum replacement (GIBCO), 10 ng/µl EGF (Sigma-Aldrich), 1 µg/ml insulin (Sigma-Aldrich), 0.5 µg/ml hydrochortisone (Sigma-Aldrich), 4 µg/ml bFGF (Sigma-Aldrich). When grown in adherent conditions, culture dishes were coated with 0.1% gelatin (Sigma-Aldrich) to enhance cell adhesion.

2.2.1.1 Mammosphere cultures

Human primary cells were seeded in low attachment plates at clonogenic density of 1000 cells/ml, with the same culture medium used for the adherent culture. After 7 to 10 days in culture, mammospheres were collected, counted and seeded on a feeder layer of human foreskin at early passage (Stemgent).

2.2.2 Feeder layer

Human foreskin fibroblasts (Stemgent), were grown in DMEM supplemented with 10% FBS, 50µg/ml 2-mercaptoethanol (Gibco, Invitrogen), 1X non essential amino acids mixture (Gibco, Invitrogen), were plated in 6 well at 80% confluency and mitotically inactivated with Mitomycin C (Sigma-Aldrich) for 1h and 45 minutes. Inactivation was followed by an overnight recovery in complete growth medium.

2.2.3 Cell xenografts under mouse kidney capsule

300 mammospheres or 150000 single cells derived from human primary samples were plated on mitotically inactivated human foreskin fibroblasts. After 72h in culture, sample were collected by scraping and centrifuged in order to obtain a compact pellet. Sample transplantation under the mouse kidney capsule was performed as described [21].

2.3 Cell transfection with RNA oligonucleotides

LA7 cell lines were transfected with 100 nM of miRIDIAN^{TM} Mimic has-miR-199a, miRIDIAN^{TM} Hairpin Inhibitor has-miR-199a-3p, or Negative Control#1 Mimic and Hairpin Inhibitor miRNAs (Dharmacon). Oligonucleotide transfection was performed

with INTERFERin according to the manufacturer's instructions (Polyplus). Cells were collected at 72 hours after transfection and processed.
For long-term cell treatment, cells were collected at 72 hours intervals and divided in two: replated to receive new transfection or left untreated for recovery. Every 72h cells were collected for RNA extraction or fixed for immunofluorescence analysis.
MCF7 cells were transfected with 50 nM MISSION® siRNA PTPRF (Sigma-Aldrich, SASI_Hs01_00159785) or with 50 nM siRNA Universal Negative Control #1 and MISSION® siRNA Universal Negative Control #2 using INTERFERin according to the manufacturer's instructions (Polyplus).

2.4 Real Time quantitative PCR

Extraction of total RNA was obtained using the TriZol™ reagent following the manufacturer's instructions (Invitrogen). Quantification of mature miRNAs was achieved using the TaqMan MicroRNA Assays listed in Table 2.1 according to the manufacturer's instructions (Applied Biosystems).

SPECIES	MIR ID	Taq-Man Assay ID	MATURE SEQUENCE
human	hsa-miR-199a-5p	000498	CCCAGUGUUCAGACUACCUGUUC
	hsa-miR-199a-3p	002304	ACAGUAGUCUGCACAUUGGUUA
	hsa-miR-214	002306	ACAGCAGGCACAGACAGGCAGU
	hsa-miR-200c	002300	UAAUACUGCCGGGUAAUGAUGGA

***Table 2.1**. TaqMan MicroRNA Assay used for RT-qPCR*

RNA was reverse transcribed using High Capacity RNA to cDNA Master Mix (Applied Biosystems) and mRNA expression was measured by RT-qPCR with SYBR Green chemistry (Applied Biosystems).

species	gene	Gene bank ID	FW	RV
rat	Cdh1	NM_031334	TCAGGACCAGGACTACGATTAT	CCACACAGGAACGACTCTCT
	Twist1	NM_053530	CAGAGATTCCCAGAGGCAAC	AAAATAAAACATTCTTCGTCAAAAGG
	Vim	NM_031140	ATTTCTCTGCCTCTTCCAAACTT	CCGTCTTAATCAGGAGTGTTCTT
	PTPRF	NM_019249.1	TGAGAGCAGCCCGGTACT	GAGGCTCAACCTCCACCTT
	Snai1	NM_053805	TCCACAAACACCAAGAGTCTG	CCAGGGAGATGCCAGTGAG
	Snai2	NM_013035	CCATTAGTGACGAAGAGGAGAG	CCAGACCAGAGAAAGTAGAATAGG
	Tcf3	NM_001107865	GCCTACCACTGCCTTCAAGA	CTGCCTGCCACTCTGAGAC
	Twist2	NM_021691	GACCAAGGCTCTCAGAACAAG	CAGGAGTGTGCGGGTAAGA
	Zeb2	NM_001033701	CAACTCTGATGAACTGCTGAAAA	GTGATGGTGACCTGGAACTG
	Fn1	NM_019143	GCAGGCTGACAGAGATGATTC	GTGTGGATTGACCTTGGTAGAG
	Foxc2	NM_001101680	TGTAAACGAGTGCGGATTTGT	AGTAACAGTTGGGCAAGATGAA
	Hprt1	NM_013556	TCCATTCCTATGACTGTAGATTTTATCAG	AACTTTTATGTCCCCCGTTGACT
mouse	Cdh1	NM_009864	TCCTGGGCAGAGTGAGATTTG	TCTGTCGCCACTTTGAATCG
	Hprt1	NM_013556	TCCATTCCTATGACTGTAGATTTTATCAG	AACTTTTATGTCCCCCGTTGACT
human	CDH1	NM_004360	CGACCCAACCCAAGAATCTATC	CTTGGTCTTTATTCTGGTTATCCATGA
	PTPRF	NM_002840.3	CACGACAGACACCCGCTTTA	CGCGGACCTTGATGTCGTA
	HPRT1	NM_000194	TTTGCTGACCTGCTGGATTACA	GGTCATTACAATAGCTCTTCAGTCTGAT

***Table 2.2**. Primers used for RT-qPCR*

2.5 Immunofluorescence assay

LA7 cells were fixed in 4% PFA for 10 minutes at room temperature. Cells were then incubated overnight at 4°C with the following primary antibodies: monoclonal anti-α-Smooth Muscle Actin (Sigma-Aldrich), monoclonal anti-β-catenin (Sigma-Aldrich), SNAI1 (H-130) (Santa Cruz Biothecnology), SLUG (G-18) (Santa Cruz Biotechnology), CDH1-FITC conjugated (BD) or Phalloidin-FITC conjugated F432 (Invitrogen). Secondary antibodies Goat Anti Mouse 488 (Alexafluor-Invitrogen), Donkey anti Rabbit 594 (Alexafluor-Invitrogen) or Rabbit anti Goat 488 (Alexafluor-Invitrogen) were then incubated 1h at room temperature. Nuclei were stained with Hoechst dye 33342 (Sigma-Aldrich).

2.6 In situ hybridization

miR *in situ* hybridization assay were carried out as described [123] using miRCURY LNA detection probe 3841-08 for both strands of the miR-199a (Exiqon). For detection signal alkaline phospatase conjugated anti-fluorescein antibody fragment (Fab) (Roche) was incubated and colorimetric reaction was achieved as described [124].

2.7 Lentiviral particle production and transduction.

2.7.1 Constructs production: overexpression

The miR-199a-3p mature sequence (MIMAT0000232) was cloned into the pcDNA^{TM}6.2-GW/EmGFP-miR plasmid (Invitrogen) using the BLOCK-iT^{TM} Pol II miR RNAi Expression Vector Kit (Invitrogen) following the manufacturer's instruction with the following custom-synthesized oligos: mir-199a-3p_TOP: 5'-TGCTGACAGTAGTCTGCACATTGGTTAGTTTTGGCCACTGACTAACCAATGCA GACTACTGC-3' and mir-199a-3p_BOTTOM:5'-CCTGACAGTAGTCTGCATTG GTTAGTCAGTCAGTGGCCAAAACTAACCAATGT GCAGACTACTGTC -3'. The miR-199a-3p sequence was amplified from this vector together with the flanking regions needed for the correct processing by Drosha, using the following primers FW: 5'-GGCATGGACGAGCTGTACAA-3' and RV_NotI: 5'-GTGCGGCCG CATCTGGGCCATTT-3' in order to add a NotI restriction site at the 3' end of the amplicon. The PCR product was then cloned (using BamHI and NotI) into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (cat. #CD511b-1, SBI).
MIR-199a precursor with flanking regions for Drosha-mediated processing was amplified from MCF7 genomic DNA using the primers inf_199a_pCDH-Eco_F (5'-**TAGAGCTAGCGAATTC**TGCCACGTCAGAAGGGACA-3') and inf_199a_pCHD-Eco_R (5'-**ATTTAAATTCGAATTC**TCTTCTATGCGAGGCTCTGC-3'). Those primers add the suitable sequnces (in bold) for cloning the PCR product into the pCDH-CMV-MCS-EF1-copGFP in the EcoRI restriction site by using the In-Fusion™ Advantage PCR Cloning Kit (Clontech).

2.7.2 Constructs production: decoys

The decoy sequences were designed as a chain (called concatamer) of short sequences complementary to the each strand of the miR-199a. The oligos designed to block miR-199a-3p (DECOY-199a-3pTOP**:** 5'-GCATCTGTCATCAGACACTAACCAATTCAGTGTCATCAGACACTAACCAAT-3' and DECOY-199a-3pBOT:5'- GATGCATTGGTTAGTGTCTGATGACACTGAATTG GTTAGTGTCTGATGACA-3') were purchased with phosphorylated 5' ends and were annealed at the concentration of 40 µM each in an annealing buffer (10 mM Tris pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA), 4min at 95°C followed by 15min at room temperature. The concatamerization was performed with 2µl of annealed oligos in a total volume of 20 µl using 1 µl of T4 DNA ligase (400 U/µl), incubating at 37°C for 15 min and then overnight at 16°C. The ligation product was checked by electrophoresis on a 2.5% agarose gel. The portion of the resulting smear of about 1kb was sliced from the gel, purified using NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, #740609). In order to use a topoisomerase-based PCR cloning system, the DNA is dephosphorylated with alkaline phosphatase and purified using NucleoSpin® Gel and PCR Clean-up (Machery-Nagel, #740609) following manufacturer's protocol. Concatamers were eluted in 15 µl and a "fill-ends" reaction was performed using Taq polymerase (Platinum®Taq DNA Polymerase, Life Technologies, #10966-018) in 20 µl at 72°C for 10 minutes. The 5'-protruding termini of the concatamers are used as template to form complete double-stranded ends with adenosine (A) nucleotide protruding at the 3' due to the terminal tranferase activity of the Taq polymerase; allowing for the cloning with TOPO® TA Cloning® Kit (Life Technologies, #450641). Using In-Fusion™ Advantage PCR Cloning Kit (Clontech), the miR-decoy was then cloned into the pCDH-CMV-MCS-EF1-copGFP (System Bioscience, # CD511B-1) from the pCRII shuttle vector with modified M13 primers (M13 INFUSION-FW-TAGAGCTAGCGAATTCTGTAAAACGACGGCCAGT and M13 INFUSION- Rv-ATTTAAATTCGAATTCCAGGAAACAGCTATGACC) following manufacturer's instruction.

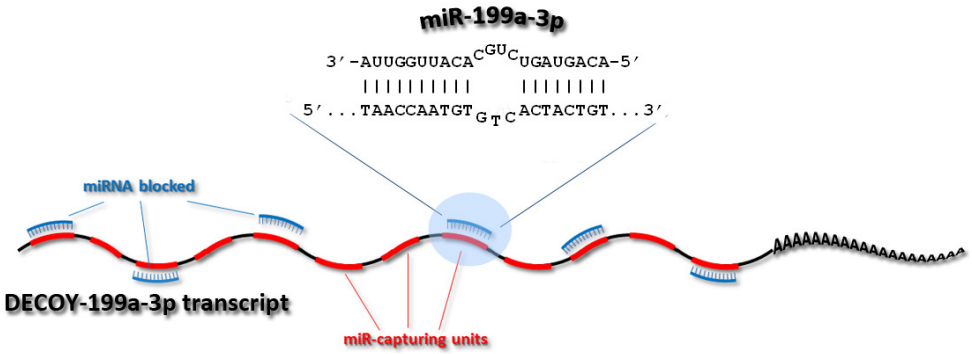


Figure 2.1. Schematic representation of a miR-decoy sequence action.
Decoys are designed as concatamer of miR-capturing sequences that distract miR from its physiologic targets.

The decoy was cloned into the lentiviral vector in both orientation allowing us to obtain either decoy for miR-199a-3p and miR-199a-5p. The figure 2.1 illustrates the annealing of miR-199a-3p to decoy "miR-capturing" units.

2.7.3 Lentivirus production

The lentiviral particles were produced in HEK293T cells by transfecting the pCDH vectors obtained as previously described, together with psPAX2 and pMD2.G as helper vectors (with a ratio 3:2:1 respectively) using the Lipofectamine™ 2000 Transfection Reagent (Life Technologies, #11668-019) following manufacturer's instructions.
The cell culture supernatant containing the lentiviral particles, harvested after 48 and 72 hours, was concentrated with the PEG-it™ Virus Precipitation Solution (System Biosciences, #LV810A-1) following manufacturer's instructions.

2.7.4 Transduction

After determining lentiviral titration by cytofluorimetry, epithelial cell lines were transduced with a multiplicity of infection (MOI) of 8 in order to obtain almost 100% of cells expressing the desired construct. The appropriate amount of virus preparation was added to the culture medium, supplemented with Hexadimethrine Bromide (Sigma-Aldrich) at the final concentration of 8 µg/ml. After over-night incubation, the transduction medium was replaced with fresh medium.
For primary cells transduction, the appropriate amount of the lentiviral suspension was added to a 100000 cells/ml suspension in a 1.5 ml tube and centrifuged at 800rcf for 30 minutes at room temperature. The surnatant was then discarded and cells were resuspended in full fresh medium.

2.8 In silico target gene identification

Putative targets of miRNAs were predicted *in silico* and analyzed using the following softwares: TargetScan 5.0 (http://www.targetscan.org/), miRBase (http://microrna.sanger.ac.uk/) and Pictar-Vert (http://pictar.mdc-berlin.de/). Lists of targets were further analyzed and functionally clustered using the functional annotation database David (http://david.abcc.ncifcrf.gov/).

2.9 Reporter assay

The 3' untraslated regions (3'UTR) of miR putative target genes were cloned downstream of the Renilla luciferase gene in the psiCHECK2 plasmid (Promega) that also contains Firefly luciferase coding sequence. After PCR amplification with ad hoc forward (FW) and reverse (RV) designed primers:

FW: 5'-GCCTCGAGCCAAGGTGAATAGCACA-3' and RV: 5´-TAGCGGCCGCTAAAACAGCTATGCAC-3´ containing a XhoI and NotI restriction site respectively, the fragment of 3'-UTR of the human *PTPRF* gene (NM_002840), spanning 300 bp around the putative miR binding site was cloned.
LA7 cells were transduced with miR-199a-3p lentiviral particles at MOI 8 and after 48h, 15000 cells were transfected with 10 ng of the psiCHECK2 plasmid with the desired 3'UTR sequence or without (ctrl) using the lipofectamin reagent (Invitrogen) following the manufacturer's instruction. After 48 hours, cells were lysed and the two luciferase signals were detected by the Victor Light 1420 Luminescence Counter (Perkin Elmer) using two sequential bio-luminescence reactions generated by the Dual-Luciferase Reporter Assay System (Promega). The renilla luciferase measurement was carried out using the firefly luciferase for internal normalization.

3.RESULTS

3.1 LA7/LA7E is a model of breast cancer progression

3.1.1 Tumors generated by LA7 are heterogeneous

The rat cell line LA7 is a model of mammary gland carcinogenesis since, when engrafted in immunodeficient mice, single cells recapitulate the entire process of tumor development including metastasis formation.
After the injection of LA7 in NOD/SCID mice, heterogeneous tumor is formed in which different populations of cells can be individuated. Immuno-histochemical analysis of LA7-induced tumors shows a gradient of differentiation from the core of the tumor mass to the periphery. In the center of the tumor mass differentiated structures and functional secretory tubules are present (fig. 3.1 up), with a large number of cells expressing the mammary gland lineage specific markers: cytokeratins K14 or K18 [121]. In this area, cells arrangement is indeed reminiscent of the derivation of LA7 from epithelial tissue, as shown by E-cadherin and β-catenin staining at the cell boundaries. In contrast, the peripheral region of the tumor is highly undifferentiated and lacks tridimensional structures that are reminiscent of the mammary gland architecture (fig. 3.1 bottom).

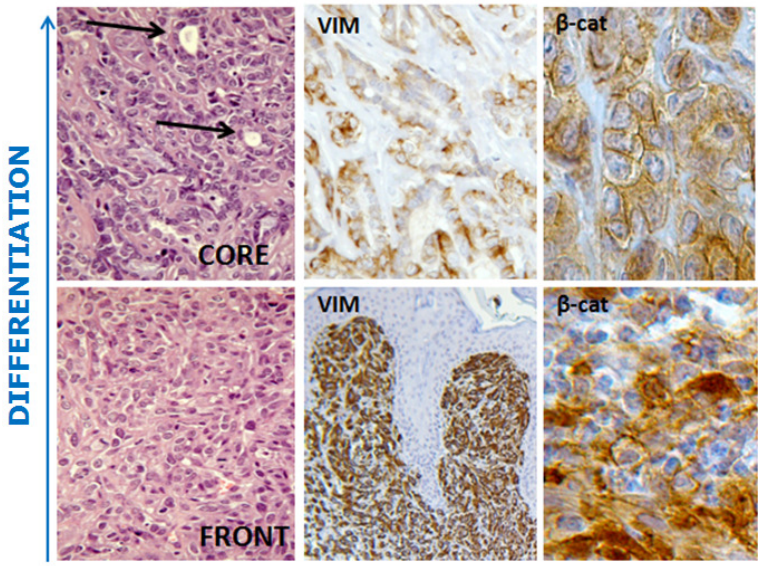


Figure 3.1. Tumor induced in NOD/SCID mice by a single LA7 is composed by different cell populations. *Immuno-histochemical analysis of tumor section shows a gradient of differentiation from the core (more differentiated) to the front and acquisition of invasion-associated markers at the front (arrows highlight tubular structures; VIM: vimentin; β-cat: β-catenin)*

Moreover the tumor-stroma interface contains a great number of cells positive for the mesenchymal marker Vimentin in which β-catenin assumes a nuclear localization. The latter are markers of cells that have acquired invasion-associated abilities through stimulatory action of the surrounding inflammatory environment and the reactive stroma.

3.1.2 LA7E have signature of invasive cells

After dissociation of the tumor mass induced in NOD/SCID mice by the injection of a single LA7, distinct populations of cells were isolated on the basis of their morphology. From the same tumor, a population of epithelial cells that recapitulate features of the parental LA7 (LA7-like cells, sLA7), cells with elongated and fibroblast-like morphology (LA7E cells) and a population of mesenchymal like cells were isolated (fig.3.2).

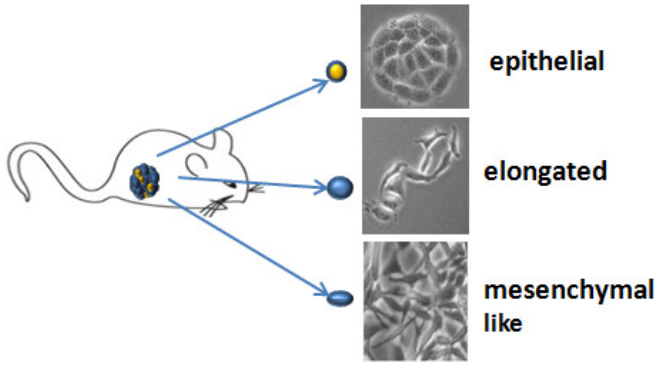


***Figure 3.2**. Morphology of cell populations isolated from a LA7-induced mammary gland tumor.*

To characterize the different populations of cells obtained in confront to tumor founder LA7 or to LA7 treated with differentiating agents (*i.e.*, DMSO), microarray based analysis were conducted both at the mRNA and microRNA level.
The LA7E signature does not overlap with that of the epithelial hierarchy of cells made after induction of differentiation in LA7 epithelial cells. 2511 genes were found significantly differentially expressed between sLA7 and LA7E. The group of genes differentially expressed was found to be enriched for genes related to cell adhesion, motility, wound healing and cytoskeleton organization (tab. 3.1).
Our hypothesis is that the formation of LA7E during breast cancer progression is induced by the local tumor environment and that this is a required step for the tumor evolution towards metastasis.

GOid	name	refnum	interestnum	pvalue	adjustp
GO:0009611	response to wounding	687	166	8.87E-13	4.06E-10
GO:0042060	wound healing	275	82	1.53E-11	4.06E-09
GO:0016477	cell migration	724	166	8.17E-11	1.99E-08
GO:0030030	cell projection organization	756	166	2.57E-09	4.52E-07
GO:0007155	cell adhesion	679	151	5.76E-09	8.68E-07
GO:2000145	regulation of cell motility	389	96	2.24E-08	2.94E-06
GO:0031589	cell-substrate adhesion	180	50	1.50E-06	0.000105
GO:0051493	regulation of cytoskeleton organization	229	58	5.75E-06	0.000357
GO:0034330	cell junction organization	114	33	3.52E-05	0.001632
GO:0032886	regulation of microtubule-based process	94	28	7.73E-05	0.003161
GO:0030198	extracellular matrix organization	140	36	0.000233569	0.007658
GO:0022408	negative regulation of cell-cell adhesion	25	10	0.001421881	0.032476

Table 3.1. Enrichment analysis of mRNA differentially expressed between sLA7 and LA7E cells*. (GOid: Gene Ontology Identifier; Refnum: number of reference genes in a GO term; Interest number: the number of interesting genes in a GO term; Adjustp: resulting p-value corrected using the false discovery rate (FDR) method)*

3.1.2.1 Markers of invasiveness are differentially expressed in the LA7 model of tumor progression

Giving to the gene signature, to the enrichment analysis showing a component of genes differentially expressed taking part of important biological processes involved in tumor progression, and to the fibroblastoid morphology, we investigated if LA7E are the candidate cells needed by the tumor to progress in the metastatic cascade.

To this end, we proceeded with the validation of expression data of the genes that were found modulated by microarray analysis and that represented established markers of invasiveness and migratory abilities. RT-qPCR and immuno-fluorescence assays were performed to confirm expression both at mRNA and at protein levels. Both the transcripts and proteins Twist1 and Snai2, known transcriptional regulators of the CDH1 gene coding for E-cadherin, were up-regulated in LA7E compared to LA7 cells. Even though the transcription level of Snai1 is higher in LA7 cells, only LA7E display nuclear localization of the Snai1 protein while it localizes in the cytoplasm of LA7 cells, most likely due to ubiquitin-dependent degradation. As a consequence, LA7E cells show loss of E-cadherin and β-catenin at the membrane adhesion complexes with the concomitant nuclear accumulation of β-catenin. Moreover, α-SMA and Vimentin are enriched in LA7E, which are typical traits of a motile cell reactive cytoskeleton (fig.3.3).

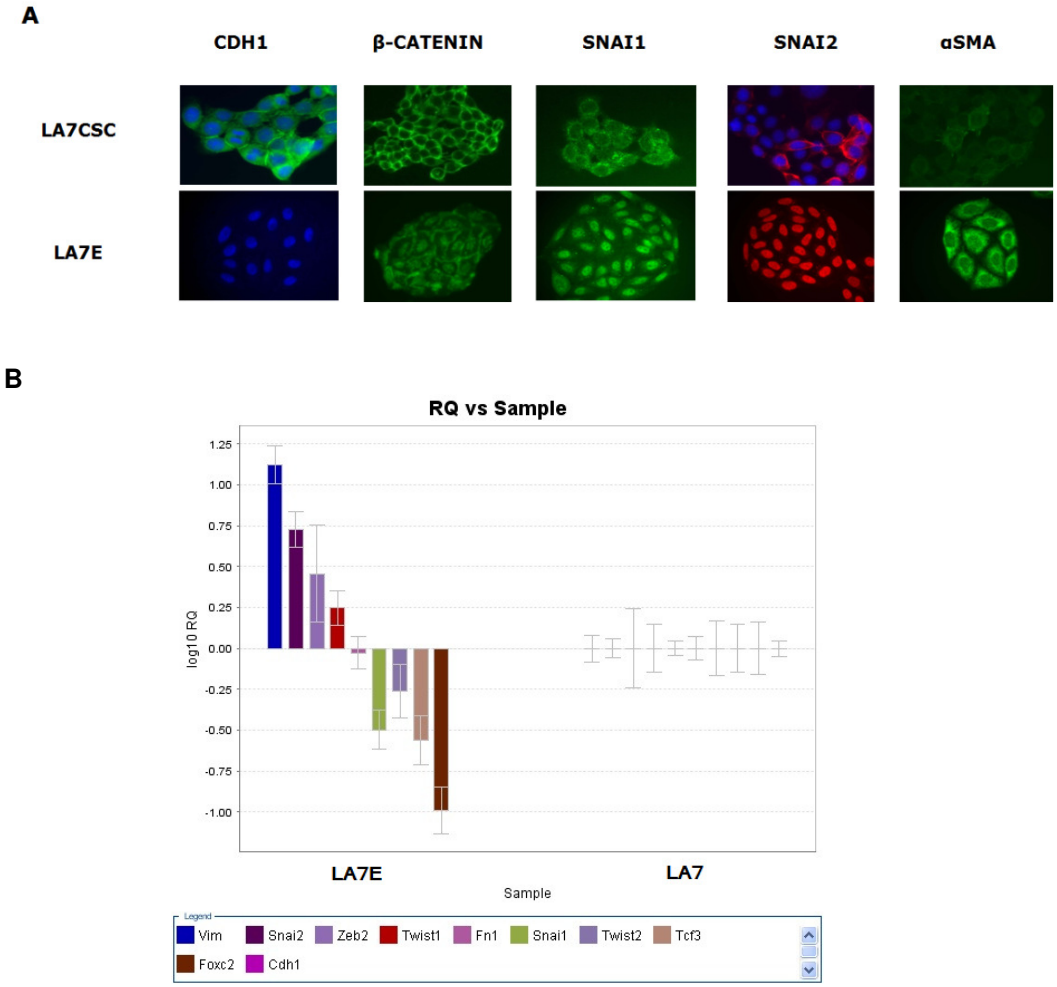


***Figure 3.3**. **A** Immuno-fluorescence staining of LA7E shows loss of epithelial marker E-Cadherin, gain of mesenchymal marker α-SMA and nuclear translocation of β-Catenin, Snai1 and Snai2. **B** Quantitative RT-PCR showing expression of known transcription factors and markers linked to invasion and migration ability (Vim: vimentin; Fn1: fibronectin; TCF3: E47; hPRT gene was used as endogenous control. Error bars indicate a confidence interval of 95%)*

3.1.2.2 MiRs are differentially expressed in LA7 system

To resolve the role of miRNAs in the biological process responsible for LA7E production and cancer progression, the expression of miRNAs that were found differentially expressed by microarray analysis was validated by RT-qPCR. Among the miRs that resulted to be differentially expressed we focused on miR-199a, whose expression was already shown to be correlated to cancer progression.

Both strands of miR-199a (*i.e*, miR-199a-3p and miR-199a-5p) are up-regulated in LA7E compared to LA7. Moreover miR-200 family members, well known gatekeepers of epithelial phenotype, resulted to be inversely correlated to the miR-199a (fig.3.4).

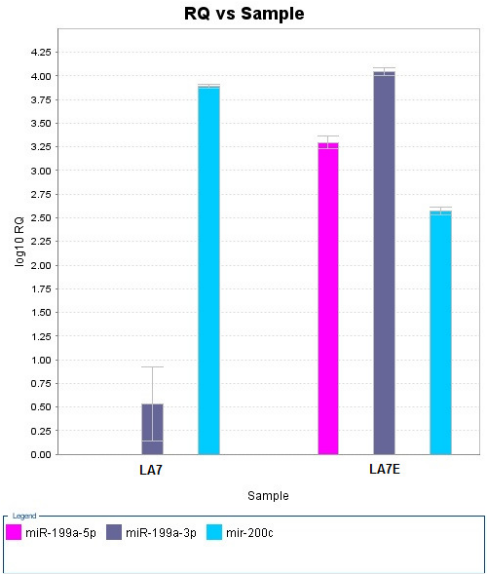


Figure 3.4. Both strands of miR-199a are up-regulated in LA7E cells compared with LA7. *RT-qPCR analysis of miR-199a compared to the expression of miR-200c. (Relative quantification is carried out in comparison to NMU-MG cells. U6 non coding RNA was used as endogenous control. Error bars indicate a confidence interval of 95%).*

3.2 miR-199a establishes the elongated phenotype in LA7

To investigate the possible role of miR-199a in the acquisition of the "elongated" phenotype by LA7 occurring during tumor progression, we up-regulated miR-199a by mimicking its activity with synthesized oligonucleotide miR analogs (MIMIC) in LA7 growing in non differentiating condition. The control samples grew in monolayer as epithelial clusters with a typical cobblestone morphology whereas the miR-199a-3p up-regulation resulted in loss of cell-cell contacts and in acquisition of fibroblast-like morphology in 72h (fig.3.5). Since the effect on cell morphology was apparently transient, we assessed whether long term exposure to miR-199a-3p was able to induce an irreversible phenotypic switch. LA7 cells were transiently transfected with miR MIMIC over a 15 days' time course. Every 72h cells received a new dose of MIMIC or were left untreated for recovery. After 72h, it was observed that LA7-miR-199a-3p cells were able to revert to their former epithelial morphology and marker profile as indicated by CDH1 expression.

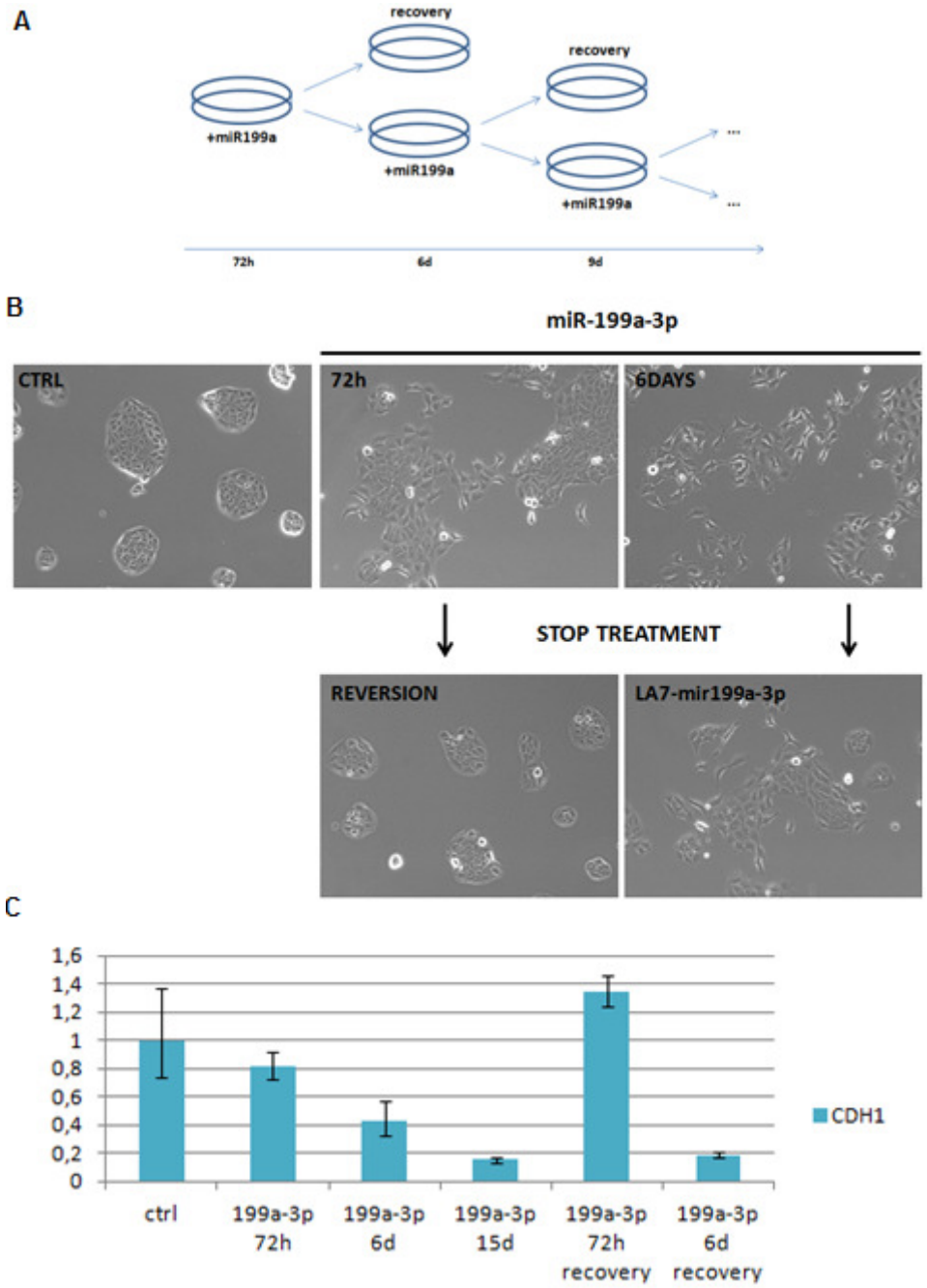


Figure 3.5. MiR-199a-3p establishes the elongated phenotype in LA7 cells. A *Schematic representation of the experimental procedure.* ***B*** *Long-term miR-199a-3p treatment of LA7 leads to morphological switch and retention of fibroblast-like phenotype after oligos deprivation.* ***C*** *RT-qPCR: Epithelial marker CDH1 is down-*

regulated after miR-199a-3p ectopic expression resulting in an irreversible cell change after 6 days of treatment (72h recovery: 72h trasfection followed by 6 days of recovery; 6d recovery: 6 days transfection followed by 6 days of recovery. The hPRT gene was used as endogenous control. Error bars indicate a confidence interval of 95%).

After 6 days the switch is irreversible and the epithelial cobblestone growth pattern could not be completely reestablished (fig.3.5A and B) nor could the expression of epithelial marker CDH1 be reacquired. This result indicates that miR-199a-3p up-regulation is sufficient to induce the phenotypic switch and that this effect is reversible until a critical threshold is overcome.
To assess whether, in addition to the observed morphologic modifications, miR-199a-3p expressing LA7 also changed at the molecular level, we measured markers level in comparison with untreated and MIMIC-negative control transfected cells. Up-regulation of miR-199a-3p induced the expression of Twist1, Snai2 and Vimentin and the down-regulation of E-cadherin and β-catenin. Moreover the cytoskeletal actin fibers were rearranged from a cortical to a stress-fiber pattern. In contrast none of the mesenchymal markers were up-regulated and CDH1 expression remained unaffected in untreated (not shown) or in MIMIC-negative control (Fig.3.6A and B).

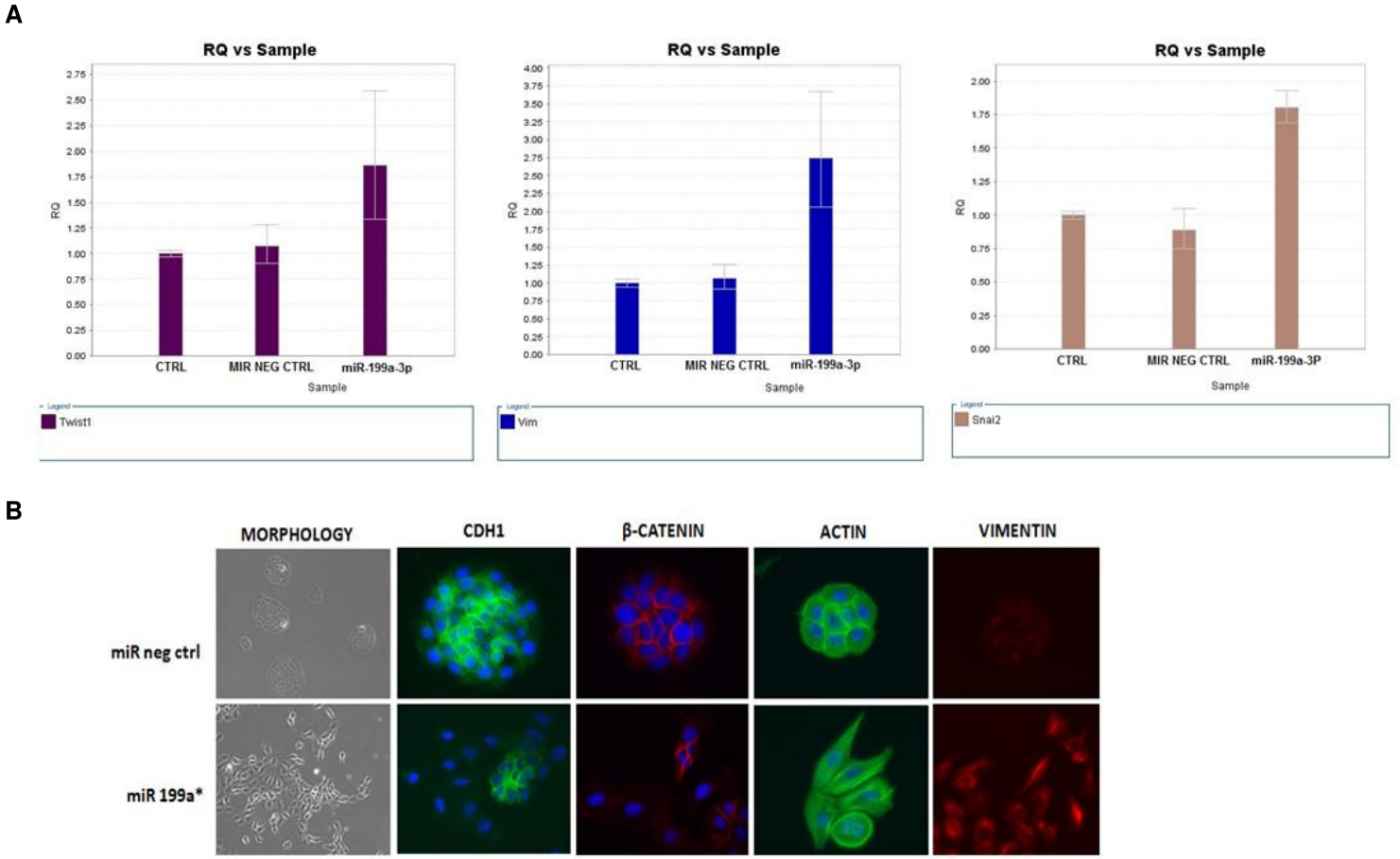


Figure 3.6. A *RT-qPCR show up-regulation of Twist1, Vimentin and Snai2 caused by ectopic expression of miR 199a-3p* ***B*** *Morphology and immuno-fluorescence staining of LA7 after ectopic expression of miR-199a-3p showing loss of cell-cell adhesion proteins and acquisition of fibroblastoid cytoskeleton.*

3.3 MiR-199a-3p regulates cell motility in LA7E cells

LA7E cells show high motility and invasive features when embedded in tridimensional scaffolds that recapitulate the extracellular matrix. We hypothesized that miR-199a-3p is necessary for LA7E invasiveness and that its expression promotes cell migration. To this end, we compared the motility of LA7E cells after down-regulation of miR-199a-3p through synthetic oligonucleotide Inhibitor transfection, with the motility of LA7E cells transfected with Inhibitor miR negative control and wild type cells in 3D collagen matrix. As shown in Figure 3.7, whereas control cells were able to migrate and invade through matrix, down-regulation of miR-199a-3p significantly abrogated the invasion capacity of LA7E.

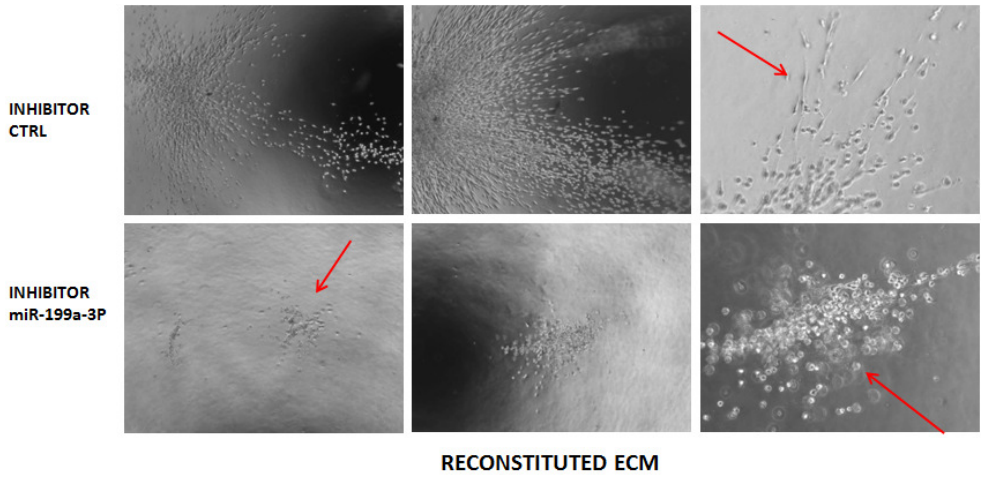


Figure 3.7. miR-199a-3p inhibition abrogates LA7E invasion ability in 3D culture. *Inhibition of miR-199a-3p led to the failure of LA7E to scatter ability in 3D collagen matrix culture. (Red arrows highlight cell morphology)*

3.4 TGF-β induces miR-199a up-regulation

Due to an EMT-like phenotypic change induced by ectopic expression of miR-199a in LA7 cells, we investigated whether miR-199a is a downstream effector of already known inducers of invasion abilities. A well established EMT model represented by the NMU-MG cell line was used for this investigation. This cell line is reported to be highly responsive to TGF-β growth factor undergoing epithelial to mesenchymal transition. Within 72h of TGF-β treatment, NMU-MG cells undergo a dramatic morphological change, from compact, cobblestone like epithelial structures to fibroblastoid spindle-shaped cells, displaying significant loss of cell-cell adhesions proteins (fig.3.8A). The morphological transition observed is coupled with the down-regulation of CDH1 and members of the miR-200 family (fig.3.8B and C).

NMU-MG cells were exposed to 10ng/ml TGF-β treatment and then collected at different time point to analyze both EMT marker and miR-199a expression.

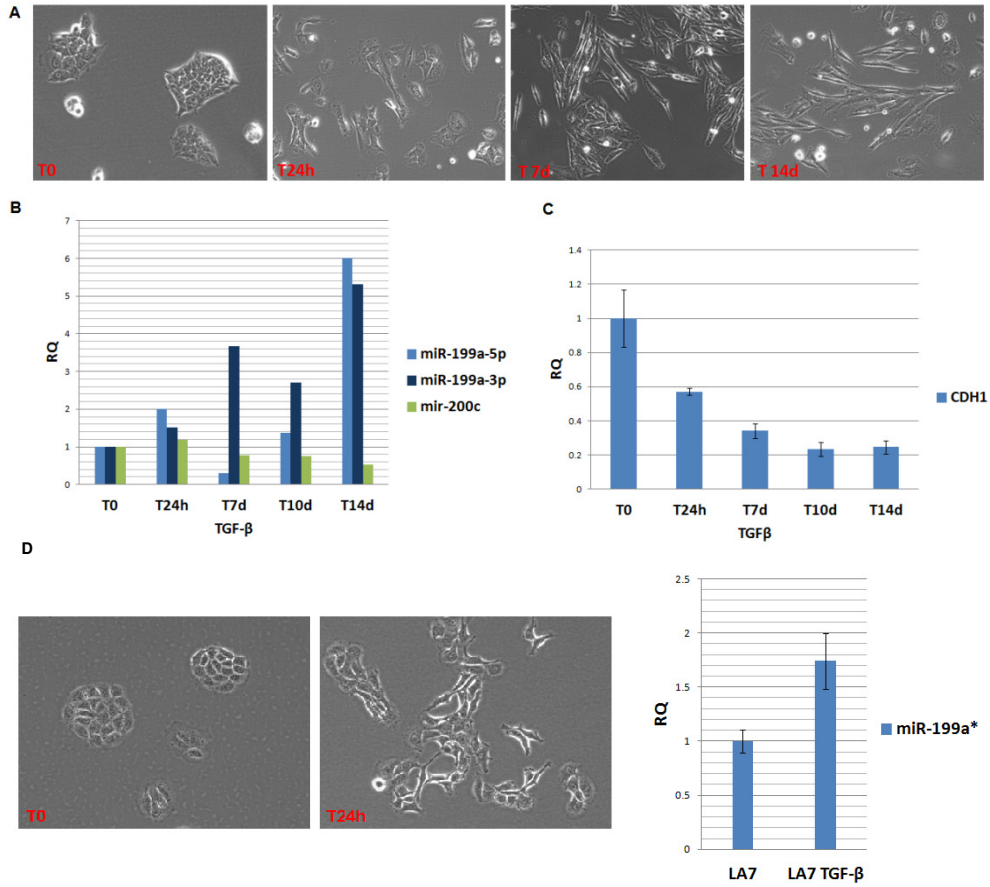


Figure 3.8. TGF-β treatment of NMU-MG and LA7 cells induces miR-199a up-regulation. A *Cell morphology is severely affected by TGF-β treatement;* ***B*** *Quantification of both strands of miR-199a and miR-200cby RT-qPCR (data are means of triplicate PCR assay. U6 ncRNA was used as endogenous control);* ***C*** *CDH1 expression time course* ***D*** *Cell morphology of LA7 and miR-199a-3p expression after 24h of TGF-β treatement (hPRT gene was used as endogenous control. Error bars indicate a confidence interval of 95%).*

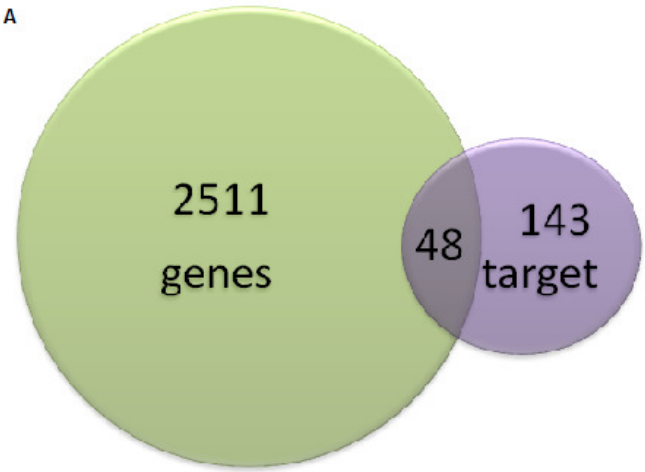
miR-199a expression is consistently increased during TGF-β stimulation and its expression is inversely correlated with miR-200c and CDH1 mRNA (fig. 3.8).

Similarly TGF-β stimulation of LA7 cells induceD morphological switch and miR-199a up-regulation (fig. 3.8D).

3.5 Identification of a new target of miR-199a-3p

To better understand the mechanism of action of miR-199a at the molecular level, we performed *in silico* predictions of putative targets using TargetScan 5.0, Miranda and PictarVert softwares. The obtained predictions were filtered with the list of genes that were found differentially expressed by microarray analysis between LA7 and LA7E, and the overlapping genes were clusterized on the base of gene ontology (fig. 3.9 B).

A



B

GOid	name	refnum	interestnum	pvalue	adjustp
GO:0042060	wound healing	275	8	1.29E-06	0.001839795
GO:0034109	homotypic cell-cell adhesion	42	4	7.13E-06	0.005989077
GO:0008283	cell proliferation	1278	14	1.12E-05	0.007096644
GO:0007155	cell adhesion	679	10	2.33E-05	0.010255675
GO:0022612	gland morphogenesis	116	5	2.35E-05	0.010255675
GO:0022610	biological adhesion	682	10	2.42E-05	0.010255675
GO:0016337	cell-cell adhesion	312	7	3.45E-05	0.011598878
GO:0022407	regulation of cell-cell adhesion	68	4	4.91E-05	0.01559443
GO:0070848	response to growth factor stimulus	249	6	8.98E-05	0.021866844
GO:0009611	response to wounding	687	9	0.000157625	0.032328765
GO:0030030	cell projection organization	756	9	0.000322669	0.048582957

Figure 3.9. A *Total number of genes differentially expressed between LA7 and LA7E (green), total number of predicted targets of miR-199a-3p (purple) and intersection of the two data sets resulting in 48 overlapping genes.* ***B*** *Gene*

ontology assisted enrichment analysis of miR-199a-3p predicted targets that are differentially expressed in LA7 compared to LA7E. (GOid: Gene Ontology Identifier; Refnum: number of reference genes in a GO term; Interest number: the number of 48 overlapping genes in a GO term; Adjustp: resulting p-value corrected using the FDR method).

Among the putative targets, Protein tyrosine phosphatase receptor like F (PTPRF) was predicted by two different algorithms (*i.e.* TargetScan 5.0 and miRANDA 4.0) to contain a seed sequence for the miR-199a-3p (fig.3.10) and for its cluster mates miR-214 and miR-3164 (not shown).

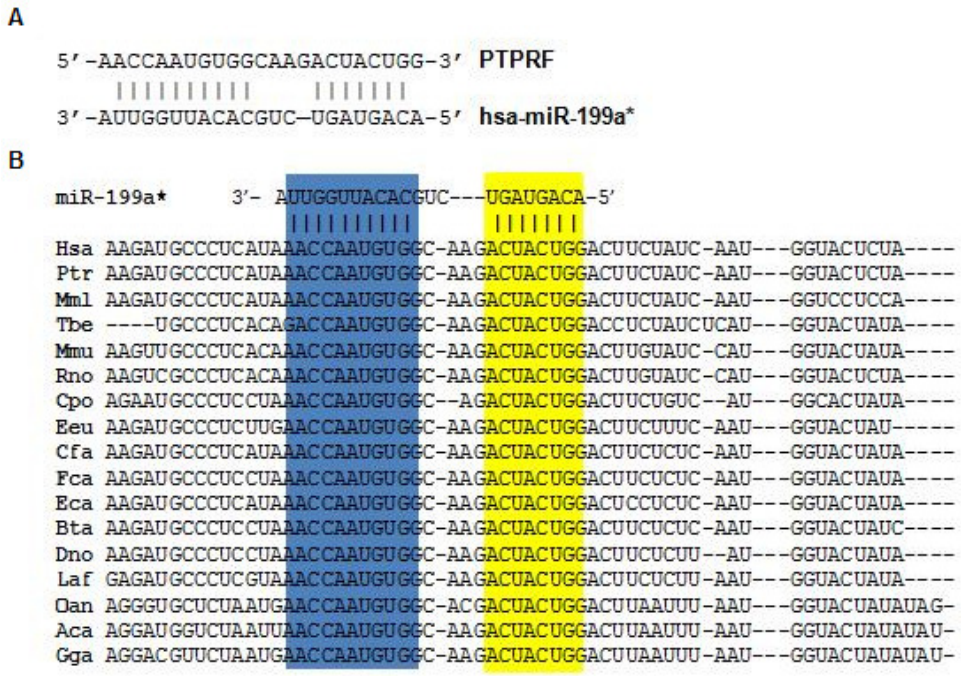


Figure 3.10. A *Pairing of miR-199a-3p to its putative target PTPRF.* ***B*** *Conservation of miR/PTPRF interaction site among species. The yellow region and the blue region show respectively the conservation of the seed region and of the 3' annealing site of miR-199a-3p among species (miR-199a*: miR-199a-3p; Has: Homo sapiens; Ptr: Pan troglodytes; Mml: Macaca mulatta; Tbe: Tupaia belangeri; Mmu: Mus musculus; Rno: Rattus norvegicus; Cpo: Cavia porcellus; Eeu: Erinaceus europaeus; Cfa: Canis familiaris; Fca: Felis catus; Eca: Equus caballus; Bta: Bos Taurus; Dno: Dasypus novemcinctus; Laf: Loxodonta Africana; Oan: Ornithorhynchus anatinus; Aca: Anolis carolinensis and Gga: Gallus gallus.)*

We demonstrated that PTPRF is post-transcriptionally regulated by miR-199a-3p using a luciferase-based reporter assay (fig.3.11). The portion of human PTPRF 3'-UTR of 300nt containing the putative miR-199a-3p binding site was cloned downstream to the renilla luciferase stop codon in the psiCHECK2 vector. LA7 cells in which permanent forced expression of miR-199a-3p or miR scrambled negative control was already induced by the lentiviral system, were transfected with psiCHECK2-PTPRF or psiCHECK2-ctrl vectors. As reported in figure 3.11, miR-199a-3p significantly reduced the luciferase activity when compared with the scrambled negative control. This result demonstrates a direct interaction of miR-199a-3p with the 3'-UTR of PTPRF that leads to reduction of its mRNA translation.

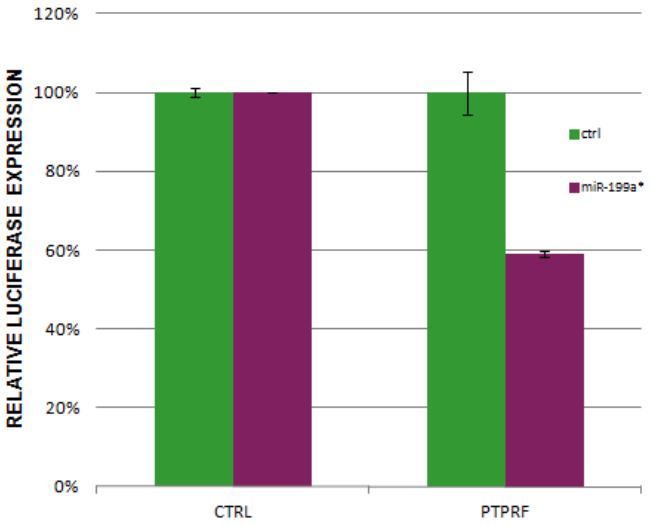


Figure 3.11. PTPRF is a target of miR-199a-3p. *The luciferase-based reporter assay demonstrates that miR-199a-3p directly interacts with the 3'UTR of human PTPRF mRNA (miR-199a*: miR-199a-3p).*

3.5.1 Ectopic expression of miR-199a-3p induces knockdown of PTPRF transcript

RT-qPCR analysis performed on LA7 transfected with MIMIC-miR-199a-3p showed a down-regulation of PTPRF mRNA level (fig. 3.12). After 72h, a slight up-regulation of PTPRF was seen, suggestive of a transient compensatory production of the transcript.

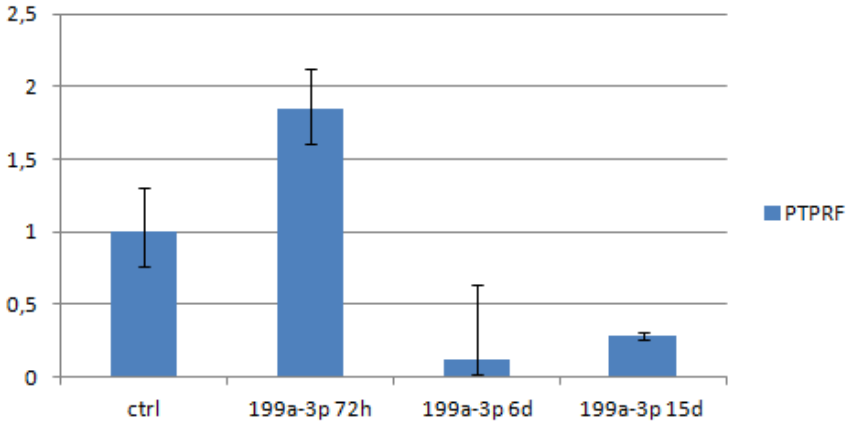


Figure 3.12 *PTPRF mRNA is down-regulated upon miR-199a-3p ectopic expression.*

Nevertheless, the prolonged up-regulation of miR lead to a permanent change in the transcriptional profile of cells that affect PTPRF production in the switching time point corresponding to the CDH1 irreversible down-regulation.

3.5.2 Knockdown of PTPRF transcript recapitulates ectopic expression of miR-199a-3p in MCF7 cells

MCF7 cells generate both in 3D matrix and in suspension cultures hollow, cyst-like polarized epithelial structure (called acini) reminiscent of mammary acinar structures in agreement with their ability to undergo luminal differentiation. Previously it was shown that PTPRF was disrupted in a human patient with syndromic amastia and that knockout mice for this protein have severe defects in mammary gland development. Accordingly, we investigated if PTPRF expression is necessary in the process of mammary gland alveologenesis. In order to ascertain this, we conducted siRNA mediated down-regulation of PTPRF in MCF7 cells that were subsequently seeded in suspension cultures. It was observed that transient inhibition of PTPRF is sufficient to effectively inhibit acini formation in about 50% of transfected cells (fig.3.13).

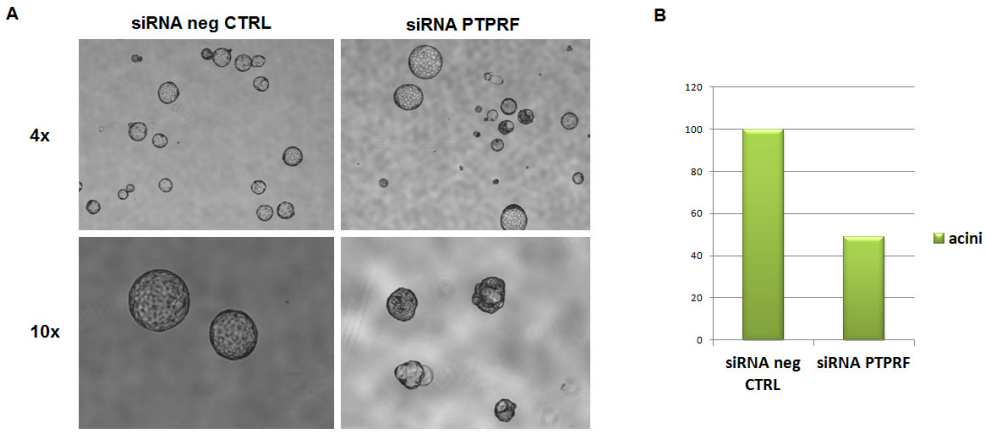


***Figure 3.13**. siRNA induced down-regulation of PTPRF results in decrease of acinar structure formation potential **A** Phase contrast pictures (4x and 10x magnification are shown), and **B** count of acinar structures (normalized with siRNA negative control MCF7 cells).*

Since PTPRF was demonstrated to be target of miR-199a-3p, we assessed whether constitutive expression of miR-199a-3p was able to recapitulate the effect that PTPRF down-regulation produces in inhibition of acinar structure formation. Cells expressing miR-199a-3p were unable to complete the production of functional acini with the same efficiency as control cells. As in the case of PTPRF knockdown, the majority of miR over-expressing cells (detected by GFP reporter expression) produced defective spherical structures in which the cavity could not be formed (fig.3.14).
To understand if there is a correlation between miR expression level and efficiency of acini formation, miR-199a expressing MCF7 were divided in two populations: functional acini were sorted from defective acini and total RNA was extracted. The miR expression level was found to be negatively correlated with acini forming efficiency. Moreover, in line to the previous observation, PTPRF was inversely correlated to miR expression and so it was higher in cells that can successfully form acini with respect to the cells that cannot (fig.3.15).

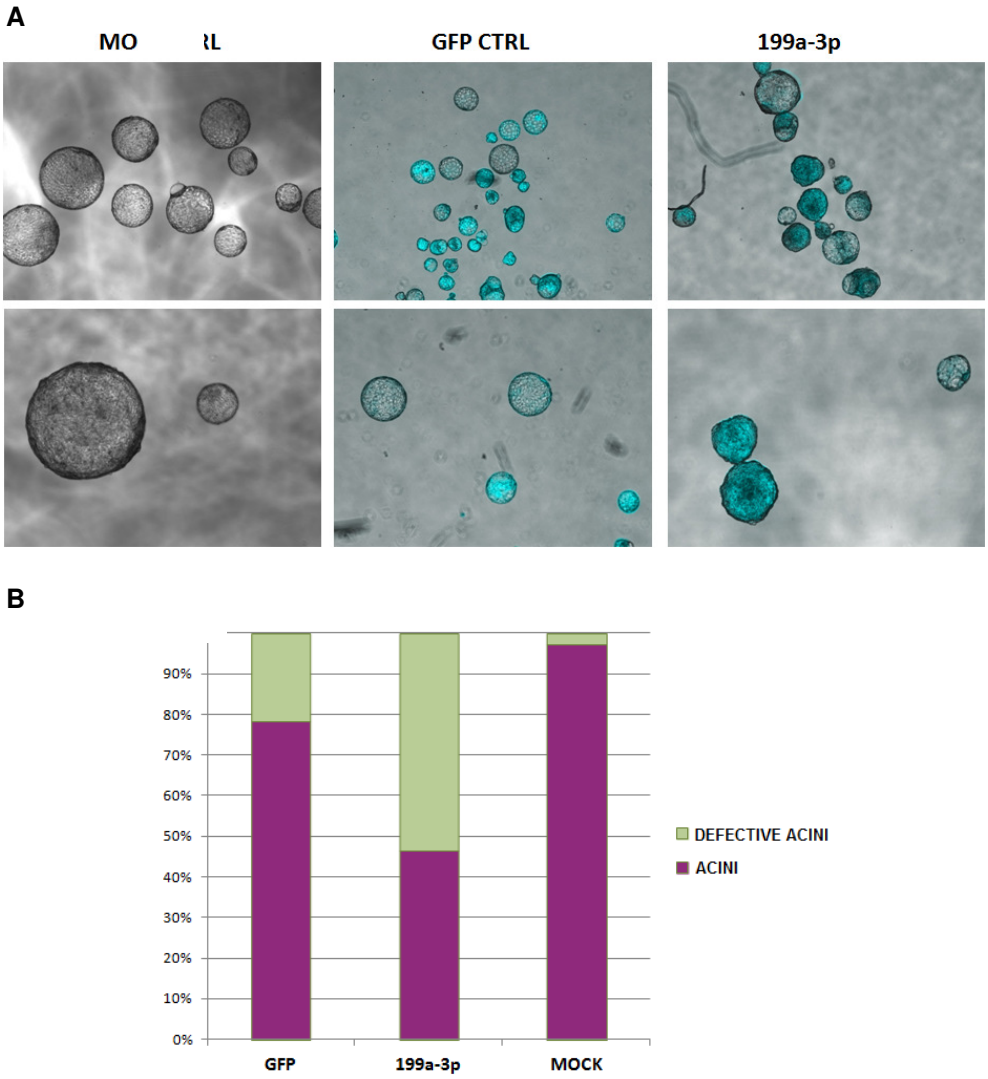


Figure 3.14. 199a-3p expressing cells are not able to efficiently complete acini formation. A *Assay showing impairment of acini formation upon miR-199a-3p up-regulation.* ***B*** *The proportion of defective acini is significantly increased upon expression of 199a-3p compared with control cells.*

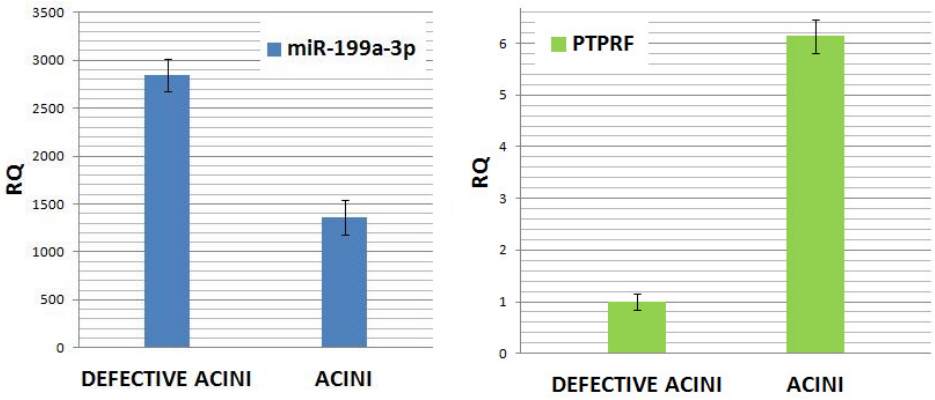
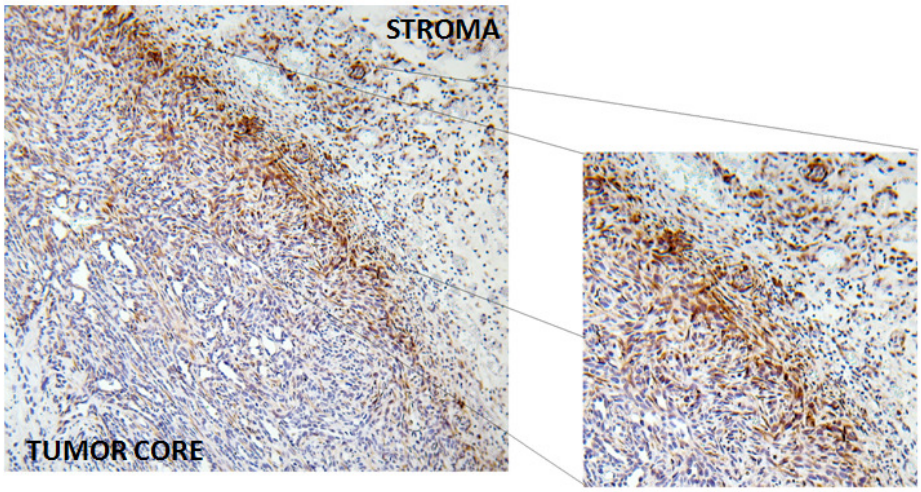


Figure 3.15. miR199a-3p expression is inversely correlated with acini formation efficiency and with PTPRF expression. *(Relative quantification in comparison to acini of the MCF7 GFP-control. U6 ncRNAand hPRT gene were used as endogenous controls. Error bars indicate a confidence interval of 95%).*

3.6 MiR-199a⁺ cells localize at the tumor-stroma interface

In situ hybridization analysis of LA7 tumors show localization of miR-199a expressing cells at the invasive front of tumors generated from sinlge LA7 cells in NOD/SCID mice (fig.3.16A). Similar results were obtained by *in situ* hybridization analysis of a cohort of human breast cancer sections and figure 3.16B shows an example.

A



B

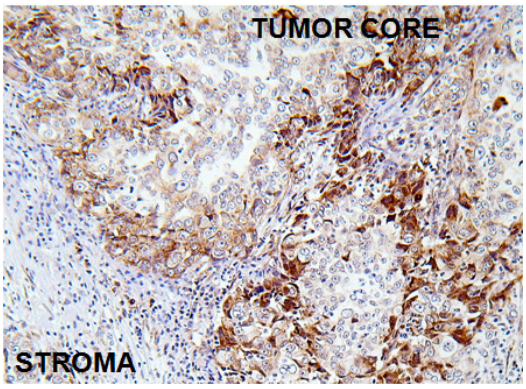


***Figure 3.16**. In situ hybridization of miR-199a shows localization of **A** LA7 cells and **B** human adenocarcinoma cells expressing the miR-199a at the tumor-stroma interface.*

3.7 In vivo analysis of miR-199a modulation

In order to verify the role of miR-199a expression during tumor development *in vivo*, stable expression of the miR was obtained in cells using a lentiviral system approach. Moreover in order to test whether the endogenous form of miR-199a, expressing two strands that are both up-regulated during tumor progression, is necessary for metastasis formation, individual inhibition of the two strand of the miR-199a was obtained through a decoy-based silencing approach stably expressed with the same lentiviral system.

3.7.1 Decoy-199a is an inhibitor of miR-199a

To verify the functionality of decoy-miR199a in the inhibition of endogenous miR-199a activity, LA7 cells were transduced with decoy-miR-199a-3p or an empty vector coupled with the copGFP protein. LA7 cells where transduced 48 hours before receiving the transfection of either miR-199a-3p mimic or a scramble mimic control at the concentration of 100 nM. A switch from an epithelial LA7 morphology to a fibroblast-like morphology was observed with miR-199a-3p, likely due to the down-regulation of its molecular targets. Cells expressing decoy-miR-199a-3p were unable to undergo miR-199a-3p mediated cellular changes (fig. 3.17). This demonstrates that the stable expression of decoy-miR is effective in blocking miR action.

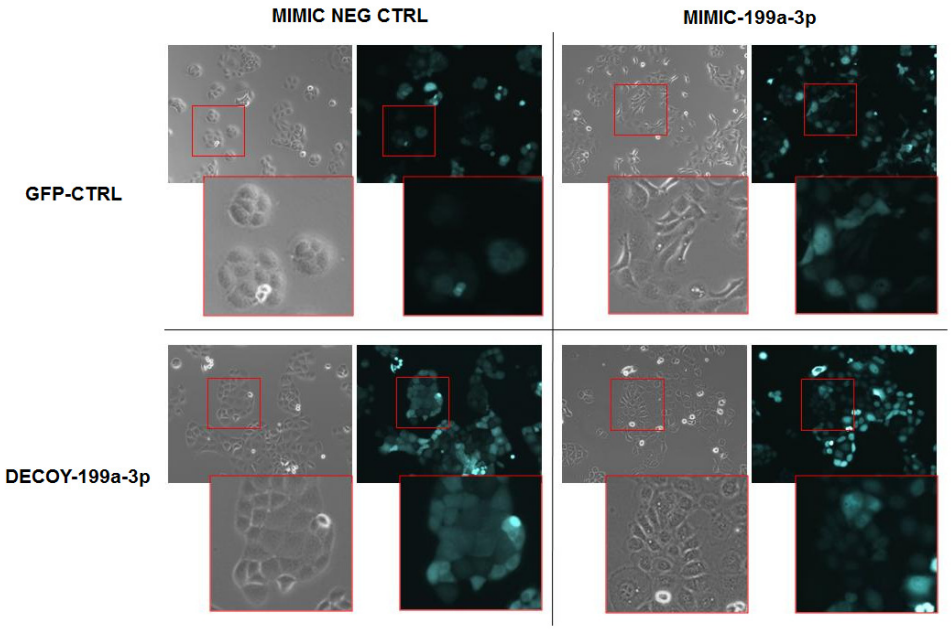


***Figure 3.17**. LA7 cells expressing GFP-decoy-199a-3p are not sensitive to mimic-199a-3p treatment as demonstrated by failure to undergo morphological changes induced by MIMIC-199a-3p oligos.*

3.7.2 *In vivo* xenografts

3.7.2.1 Mir-199a modulation in human samples

First generation spheres were obtained from cells derived from a human mammary gland adenocarcinoma (#BC42) or from human kidney adenocarcinoma (#KT1) After 7 to 10 days, 30000 cells from dissociated spheres were transduced with a MOI 8 with GFP-control, GFP-miR-199a or with one of the two decoy viral vectors and used to generate 2nd generation spheres. After 7 to 10 days, spheres were seeded on mitotically inactivated human foreskin-derived fibroblasts (fig. 3.18).
MDA-MB-231 cells were also transduced with a MOI 8 with the same constructs and after 72h were seeded on human fibroblast feeder cells. All the samples were collected by dissociating the sheet of cells from the culture plate and implanted under the kidney capsules of NOD mice. Analysis of tumor development are in progress.

3.7.2.2 LA7 engraftment

LA7 cells transduced with miR-199a or with decoy expressing viral vectors were injected into the fat pad of immuno-compromised NOD/SCID mice. Six fat pads per sample (3 mice) were injected. Table 3.2 lists the tumors and the tumor growth rates obtained after injections.

Assay	number of tumors (out of 6 fat pad)	tumor size (mm^3)	Mice with metastasis
LA7-GFP-ctrl	6	439.6	3
LA7-GFP-miR-199a	6	663.4	0
LA7-GFP-decoy-199a-3p	6	537.7	1
LA7-GFP-decoy-199a-5p	6	412.7	1

Table 3.2. Both miR-199a up-regulation and inhibition affect metastasis formation but not tumor seeding ability.

In all conditions it was observed that the injected cells were able to form a tumor mass. Despite the observed increase in proliferation, the miR-199a expressing LA7 were unable to form metastasis while the metastatic potential of decoy expressing cells was significantly inhibited. Histopathological analysis of tumors are in progress.

4.DISCUSSION

Breast cancer is the most common tumor by incidence and one of the leading causes of cancer associated death in women [48]. Preventive screening and advances in standard treatments such as surgery, radiotherapy and chemotherapy have increased patient survival, but significant progress in terms of recurrence free life-span and overall survival are yet to be achieved [64,65]. The vast majority of patient mortality can be attributed to the capacity of the breast primary tumor to evolve into metastasis [64,65]. It is thought that the invasive behavior of breast cancer cells is the main cause of poor clinical outcome, enabling tumor cells to actively egress from the main mass and invade the surrounding tissues avoiding resection and conventional therapies [66, 67].

Studies on mammary gland development and its relationship with cancer initiation and progression are under intense scientific investigation. The model employed in our laboratory is based on a rat cancer cell line, LA7, derived from a DMBA-induced mammary adenocarcinoma. Our group demonstrated that this cell line is a suitable model to study the mammary gland differentiation and a reliable model of mammary cancer stem cells [119].

Single LA7 cells have the ability to initiate a tumor and recapitulate the entire process of carcinogenesis including metastasis formation. During tumor growth and progression, the epithelial progeny of the LA7 founder cell gives rise to different populations of cells with different morphologic features, proliferative and invasive behaviors. Understanding how the different subpopulations participate to tumor progression will lead to an important understanding on tumor evolution towards metastasis and will give the opportunity to discover new prognostic factors and therapeutic targets. Despite the increase of knowledge regarding the initial steps of the metastasis cascade, how cells evolve invasive skills to evade the primary tumor and enter the systemic spread and which are the target cells of this progression, is still under debate.

Cells showing an elongated and fibroblast-like morphology (LA7E cells) were isolated from a breast adenocarcinoma obtained in NOD/SCID mice after the engraftment of a single LA7. Consistent with our hypothesis that LA7E derive from the epithelial progeny of LA7 by a trans-differentiation occurring during tumor development, these cells show fibroblast-like morphology. In addition, lack of epithelial markers (E-Cadherin and cytokeratins), gain of mesenchymal markers (Vimentin and α-Smooth muscle actin), expression of Snai1, Snai2 and Twist1 EMT inducing transcription factors and nuclear β-catenin localization characterize LA7E. Cells with similar marker expression were identified by immunohistochemical assays to be localized at the invasive front of LA7 tumors, suggesting that LA7E are located in this area and that the tumor-activated environment cross-talk may have a role in the induction of this phenotype.

Many studies have identified fibroblast-like activated cells as the cells responsible for carcinoma invasion and migration, invoking a response to stromal cells nearby the growing tumor (including fibroblasts, endothelial cells, and macrophages) as the main cause of the evolution of these cells from carcinomas [125, 126, 127].

We identified by microarray-based gene expression analysis microRNAs differentially expressed between LA7 and LA7E. We focused on miR-199a which was strongly up-regulated in LA7E cells with respect to LA7, and whose predicted targets are enriched in genes committed to invasion and migration. As expected,

miR-199a is expressed mainly by cells that are located at the tumor-stroma interface both in LA7-induced tumors and in a highly undifferentiated human breast tumor as shown by *in situ* hybridization. According with the hypothesis that the biological process responsible for the production of LA7E cells (which does not occur spontaneously during *in vitro* cultures) is dependent on the stimulation of the inflammatory environment and the reactive stroma, we investigated if miR-199a is a downstream effector of one of the pathways already associated with the invasive program. Consequently, we treated LA7 and NMU-MG cells with cytokines known to promote EMT. Among these, TGF-β treatment resulted in the induction of a fibroblastoid phenotype in both cell lines, in part recapitulating the LA7E phenotype in the LA7 cell line, as suggested by cell shape modification and marker expression. In both cell lines TGF-β stimulation led to the up-regulation of miR-199a. TGF-β is secreted by carcinoma-associated fibroblast and macrophages, together with a plethora of other growth factors, due to its cytostatic functions [128]. During tumor development, its physiologic effect is subverted by cancer cells through loss of key mediators of the growth suppressive response [129]. Moreover, Sahai and colleagues showed that TGF-β signaling can be activated for limited periods during which tumors disseminate, returning to low levels once metastases are established, thus enabling a reversible transition of cancer cells of epithelial origin to the mesenchymal phenotype [130].

It is already shown that the promoter sequence of the miR-199a cluster harbors a binding sequence (E-box) for Twist1, one of the repressor of CDH1 that is induced by TGF-β stimulation [131, 132].

While Twist1 has temporal-spatial restricted expression during normal development, it has been reported that it is constitutively expressed in some forms of cancers [133, 134, 135, 136, 137]. Similarly miR-199a has functions in development as already described [131], and in cancer as we want to demonstrate. Notably, we found that the expression of miR-199a and Twist1 are inversely correlated in LA7 cells with respect to LA7E cells, supporting a Twist1-mediated regulation of miR expression. These results suggest that TGF-β pathway induce miR-199a through Twist1 activation.

To resolve the role of miR-199a in the acquisition of the “elongated” phenotype by LA7, we ectopically expressed miR-199a in LA7 cultured in non-differentiating conditions. MiR-199a up-regulation was sufficient to induce loss of cell-cell contacts and acquisition of a fibroblast-like morphology. Interruption of the treatment at different time points demonstrated that LA7/miR-199a cells can revert to an epithelial morphology and LA7 specific marker profile until a critical threshold is overcome. Then the switch is irreversible and the epithelial cobblestone growth pattern cannot be completely reestablished. A similar response was already reported in NMU-MG cells after induction of TGF-β stimulation [138].

In addition to the observed morphology modifications, LA7 over-expressing miR-199a changed marker expression. Up-regulation of miR-199a, in fact, produced the expression of mesenchymal marker Vimentin and a switch of the cytoskeleton from a cortical to a stress fiber arrangement. Moreover Snai2 and Twist1 were up-regulated with the consequent down-regulation of epithelial adhesion proteins E-cadherin and β-catenin at the plasma membranes.

Snai2 and Twist1 are activated during embryo development, throughout gastrulation and migration of neural crest, to favor epithelial sheet disorganization and cell migration inducing epithelial to mesenchymal transition [82, 139, 140].

Furthermore, Snai2 and Twist1 are considered important factors in the activation of fat pad invasion by mammary TEBs in branching morphogenesis [34, 35]. These transcription factors are indeed thought to be main inducers of the EMT during cancer progression and organ fibrosis.
In this context, our data support the hypothesis that up-regulation of miR-199a results in an aberrant reactivation of the physiological invasive program that starts with loss of adhesion proteins in epithelial cells.
To assess how this molecular event affects cell behaviors in a tridimentional environment, we inhibited the miRNA expression in LA7E and assessed motility and invasive capacity in a bio-scaffold that mimics the extracellular matrix of the mammary gland. Down-regulation of miR-199a significantly abrogated the invasion capacity of LA7E cells in 3D cultures.
To better understand the role of miR at the molecular level, we performed *in silico* target prediction. Among the possible targets, we demonstrated that protein tyrosine phosphatase receptor like F (PTPRF) was down-regulated with ectopic expression of miR-199a. A direct interaction of the miR with the 3'UTR of PTPRF was demonstrated using luciferase-based reporter assay.
PTPRF is a transmembrane protein member of the receptor protein tyrosine phosphatase (RPTP) family. Evidence suggests that PTPRF is a central regulator of cell-cell and cell-matrix interactions. PTPRF localizes to the adherens junctions in epithelial cells and its intracellular domain directly interacts with α-Catenin and β-Catenin [141, 142, 143]. In addition, PTPRF interacts with focal adhesion-associated proteins α-liprin and Trio and other scaffold proteins, thus implicating a role in adhesion signaling and cytoskeleton remodeling [144, 145, 146, 147]. Extra cellular matrix components heparan sulfate proteoglycans as well as the laminin-entactin complex have been identified as ligands for PTPRF. Dunah and colleagues showed that in cultures of hippocampal neurons, it promotes the accumulation of cadherin-catenin complexes at the synapses to enhance cell adhesion [141]. In addition, it has been shown to regulate the tyrosine phosphorylation status of both β-Catenin and E-cadherin [142, 143, 149, 150, 151]. Tyrosine-phosphorylation of β-catenin by tyrosine-kinases like Fyn, Met, Fer or Src is a common in tumors event that leads to loss of cadherin/catenin interaction and degradation of E-cadherin, reducing the strength of adherens junctions [90, 152, 153]. In this context, the action of tyrosine-phosphatases as negative mediators of signaling triggered by receptor-tyrosine kinases has a tumour-suppressive worthiness, as the frequent inactivating mutations of PTPs in colon cancer suggest [154, 155, 156].
Inhibition of PTPRF was already shown to produce an imbalance of the E-Cadherin and β-Catenin Tyrosine-phosphorylation status that, particularly in situations where β-Catenin degradation is compromised, might result in β-catenin nuclear accumulation and Wnt pathway activation [90]. Consequently, PTPRF inhibition can be ascribed as the cause of the adherens junction deregulation that we observed in LA7 cells under miR-199a over-expression. Moreover the dissociation of AJ complexes and the consequent increase in the free β-catenin cytoplasmic pool suggest a possible role of miR-199a in Wnt independent Wnt/ β-catenin pathway activation and defines an auto-inducible regulative loop (fig.4.1). Snai2 and Twist1 are indeed targets of the canonical Wnt pathway so the effect of miR-199a on AJ proteins is synergized by the action of these two transcription factors [157].

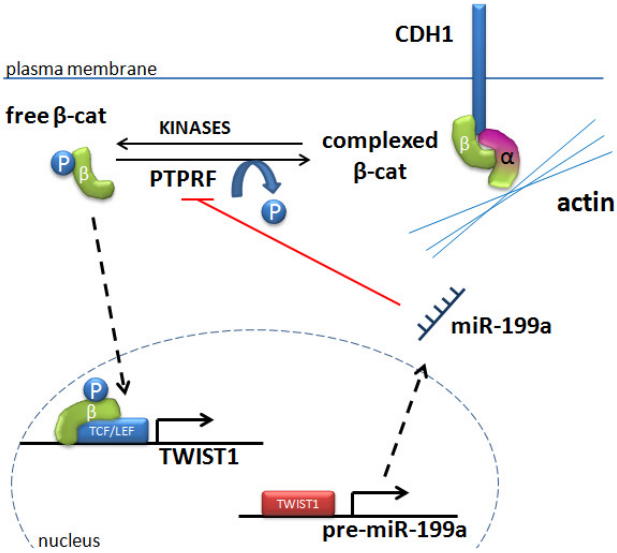


Figure 4.1. Proposed mechanism of action of the miR-199a auto-regulatory feed-forward loop. *Under miR-199a expression, PTPRF is down-regulated producing an imbalance of the Tyr-phosphorylation status of AJ proteins and consequent disruption of the membrane adhesion complex. This leads to free β-catenin cytoplasmic accumulation and eventually to nuclear shuttling of β-catenin. As a transcription factor, β-catenin induces increase of Twist1 expression which in turn promotes miR-199a transcription.*

In vivo analysis of miR-199a modulation led to the observation that miR-199a is necessary for the metastatic progression of LA7 tumor. In fact, while all the samples injected were able to form primary tumors in the mouse fat pad, none of the miR-199a over-expressing LA7 cells could form metastasis and the metastatic efficiency of cells that stably expressed inhibitor miR-199a was reduced. Since all the tumors grew at similar rates except the ones in which miR-199a over-expression was induced, for which proliferative gain was observed, the absence of proliferative potential cannot be ascribed as the cause of metastasis failure.
Carcinoma-derived metastasis express E-cadherin at the cell membrane [158, 159]. Starting with this observation and with the knowledge that cells with constitutive expression of E-Cadherin are unable to metastasize in animal models [160], it is thought that either the following occurs:

- the E-cadherin negative cells revert to an epithelial cadherin positive phenotype in order to colonize at secondary sites;
- or the E-cadherin negative cells are the pathfinder cells that allow other cells, positive for E-cadherin to migrate and seed the metastasis.

In both cases sustained expression of high levels miR-199a that is negatively regulating E-cadherin production, would lead to loss of metastatic potential of cells,

as we observed. Further investigations are needed for a better comprehension of the spatial-temporal expression of the miR during the metastatic process.
Existing evidence supports a correlation of miR-199a expression in melanoma, ovarian and gastric cancers with advanced tumor stage and poor prognosis [161, 162, 163]. In gastric cancer, miR-199a is also been proposed as a prognostic factor [164, 165].
Taken together, our results give an important contribution to the understanding of the role of miR-199a during breast cancer progression, suggest an involvement of miR-199a in the commitment of epithelial cells to EMT program and designating it as a possible linker between TGF-β and Wnt pathways.

5.BIBLIOGRAPHY

1. Woodward W.A., Chen M.S., Behbod F. and Rosen J.M. **"**On mammary stem cells". *Journal Cell Science,* 2005, 118, 3585-3594
2. Nagato T., Yoshida H., Yoshida A. and Uehara Y. "A scanning electron microscope study of myoepithelial cells in exocrine glands". *Cell tissue research,* 1980, 209:1–10,
3. Alberts B., Johnson A., Lewis J., Raff M., Roberts K., Walter P. **Molecular biology of the cell**. Garland Science, Fifth Edition 2008
4. Gjorevsky N. and Nelson C.M. **"**Integrated morphodynamic signaling of the mammary gland". *Nature Review Molecular Cell Biology* 12: 581-593
5. Hennighausen L. and Robinson G.W. "Information networks in the mammary gland". *Nature Review Molecular Cell Biology,* 2005, 6: 715-725
6. Robinson G.W. "Cooperation of signaling pathways in embryonic mammary gland development". *Nature Review Genetics* 2007, 8: 963-973
7. Wiseman B.S. and Werb Z. **"**Stromal effects on mammary gland development and breast cancer". *Science,* 2002, 296 (5570): 1046–1049
8. Green K.A., Streuli C.H. "Apoptosis regulation in the mammary gland". *Cell Mol Life Sci* ,2004, 61:1867-1883
9. Watson CJ. "Involution: Apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ". *Breast Cancer Res,* 2006, 8:203
10. Van Keymeulen A., Rocha A.S., Ousset M., Beck B., Bouvencourt G., Rock J., Sharma N., Dekoninck S. and Blanpain C. "Distinct stem cells contribute to mammary gland development and maintenance". *Nature*, 2011, 479: 189-193
11. Visvader J.A. "Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis". *Genes & Development*, 2009, 23:2563-2577
12. Yamashita Y.M, Yuan H., Cheng J. and Hunt A.J "Polarity in stem cell division: asymmetric stem cell division in tissue homeostasis". *Cold Spring Harb Perspect Biol,* 2010; 2:a001313
13. Morrison S.J. and Spradling A.C. "Stem cells and niches: Mechanisms that promote stem cell maintenance throughout life". *Cell*, 2008, 132 (4): 598-611
14. Li L. and Xie T. "Stem cell niche: structure and functions". *Annual Review Cell Developmental Biology*, 2005, 21:605-631.
15. Daniel CW, De Ome KB, Young JT, Blair PB, Faulkin LJ Jr. "The in vivo life span of normal and preneoplastic mouse mammary glands: a serial transplantation study". *PNAS*, 1968, (61): 53-60.
16. Smith GH and Medina D. "A morphological distinct candidate for an epithelial stem cell in mouse mammary gland". *J Cell Sci,* 1988, (90): 173-183.
17. Kordon EC and Smith GH. "An entire functional mammary gland may comprise the progeny from a single cell". *Development*, 1998, (125): 1921-1930.
18. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ and Wicha MS. "*In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells". *Genes Dev*, 2003, (17): 1253-1270.
19. Proia D. and Kuperwasser C. "Reconstruction of human mammary tissues in a mouse model". *Nature Protocol*, 2006, (1): 206-214.

20. Kuperwasser C., Chavarria T., Wu M., Magrane G., Gray J.W., Carey L., Richardson A. and Weinberg R.A. "Reconstruction of functionally normal and malignant human breast tissues in mice". *PNAS*, 2004, 101:4966–4971
21. Eirew P, Stingl J, Raouf A, Turashvili G, Aparico S, Emerman JT and Eaves CJ. "A method for quantifying normal human mammary epithelial stem cells in vivo regenerative ability". *Nature Medicine*, 2008, 14:1384-1389.
22. Hinck L. and Silberstein G.B. "The mammary end bud as a motile organ". *Breast Cancer Research*, 2005, 7:245-251
23. Wiseman B.S., Sternlicht M.D., Lund L.R., Alexander C.M., Mott J, Bissell M.J., Soloway P., Itohara S. and Werb Z. "Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis". *The Journal Cell Biology*, 2003, 162 (6):1123–1133
24. Larsen M., Wei C., Yamada K.M. "Cell and fibronectin dynamics during branching morphogenesis". *Journal Cell Science,* 2006, 119: 3376–3384
25. Ewald A.J., Brenot A., Duong M., Chan B.S., Werb Z. "Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis". *Dev Cell,* 2008, 14: 570–581
26. Khalil AA and Friedl P. "Determinants of leader cells in collective cell migration." *Integr Biol (Camb)*, 2010, 2(11-12):568-74.
27. Friedl P. and Gilmour D. "Collective cell migration in morphogenesis, regeneration and cancer". *Nat Rev Mol Cell Biol,* 2009, 10: 445–457
28. Ilina O., Friedl P. "Mechanisms of collective cell migration at a glance". *Journal Cell Science,* 2009,122: 3203–3208
29. Gray R.S., Cheung K.J., Ewald A.J. "Cellular mechanisms regulating epithelial morphogenesis and cancer invasion". *Current Opinion Cell Biology*, 2010, 22: 1-11
30. O'Brien L.E., Zegers M.M., Mostov K.E. "Building epithelial architecture: insights from three-dimensional culture models". *Nature Review Molecular Cell Biology,* 2002, 3: 531–537
31. Gumbiner B.M. "Regulation of cadherin-mediated adhesion in morphogenesis." *Nature Review Molecular Cell Biology*, 2005, 6: 622–634
32. Micalizzi D.S., Farabaugh S.M., Ford H.L. "Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression". *Journal Mammary Gland Biol Neoplasia,* 2010, 15: 117–134
33. Revenu C. and Gilmour D. "EMT 2.0: shaping epithelia through collective migration". *Current Opinion in Genetics & Development* , 2009, 19:338–342
34. Kouros-Mehr H. and Werb Z. "Candidate regulators of mammary branching morphogenesis identified by genome-wide transcript analysis". *Dev Dyn*., 2006, 235(12): 3404–3412
35. Lee K., Gjorevski N., Boghaert E., Radisky D.C. and Nelson C.M. "Snail1, Snail2, and E47 promote mammary epithelial branching morphogenesis". *EMBO Journal*, 2011, 30, 2662–2674
36. Baum B. and Georgiou M. "Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling". *Journal Cell Biology*, 2011, 192 (6): 907–9172011
37. Steed E., Balda M.S. and Matter K. "Dynamics and functions of tight junctions." *Trends in Molecular Biology*, 2010, 3 (20): 142–149

38. Martin-Belmonte F. and Perez-Moreno M. “Epithelial cell polarity, stem cells and cancer”. *Nature Reviews Cancer*, 2012, 23-38
39. Moreno-Bueno G., Portillo F. and Cano A. “Transcriptional regulation of cell polarity in EMT and cancer”. *Oncogene*, 2008, 27: 6958–6969
40. Zhan L., Rosenberg A., Bergami K.C., Yu M., Xuan Z., Jaffe A.B., Allred C. and Muthuswamy S.K. “Deregulation of Scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma”. Cell, 2008, 135: 865–878
41. Wodarz A. and Näthke I. “Cell polarity in development and cancer”. *Review Nature Cell Biology*, 2007, 9:1016-1024
42. Karp CM, Tan TT, Mathew R, Nelson D, Mukherjee C, Degenhardt K, Karantza-Wadsworth V, White E. “Role of the polarity determinant crumbs in suppressing mammalian epithelial tumor progression”. *Cancer Research,* 2008, 68:4105-4115.
43. Klezovitch O, Fernandez TE, Tapscott SJ, Vasioukhin V. “Loss of cell polarity causes severe brain dysplasia in Lgl1 knockout mice”. *Genes Dev,* 2004, 18:559-571.
44. Foty R.A. and Steinberg M.S. ”Cadherin-mediated cell-cell adhesion and tissue segregation in relation to malignancy”. *International Journal Developmental Biology*, 2004, 48: 397-409
45. Feigin M.E. and Muthuswamy S.K. “Polarity proteins regulate mammalian cell–cell junctions and cancer pathogenesis”. *Current Opinion in Cell Biology*, 2009, 21:694–700
46. Viloria-Petit A.M., David L., Yong Jia J., Erdemir T., Bane A.L., Pinnaduwage D., Roncari L, Narimatsu M., Bose R. ,Moffat J., Wong J.W. , Kerbel R.S., O’Malley F.P., Andrulis I.L. and Wrana J.L. “A role for the TGFβ-Par6 polarity pathway in breast cancer progression”. *PNAS,* 2009, 106 (33): 14028–14033
47. Cavallaro U. and Christofori G. “Cell adhesion and signalling by Cadherins and Ig-Cams in cancer”. *Nature Review Cancer*, 2004, 4: 199-132
48. “Breast Cancer Incidence, Mortality and Prevalence Worldwide in 2008” *GLOBOCAN 2008 (IARC)*, http://globocan.iarc.fr
49. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D. “Molecular portraits of human breast tumors”. *Nature*, 2000, 406 (6797): 747-752
50. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lønning P, Børresen-Dale AL. “Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications”. *PNAS*, 2001, 98 (19): 10869-10874
51. Sørlie T., Wang Y., Xiao C., Johnsen H., Naume B., Samaha R.R. and Børresen-Dale A.L. “Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms”. *BMC Genomics*, 2006, 7: 127
52. Ben-Porath I., Thomson M.W., Carey V.J., Ge R., Bell G.W., Regev A. and Weinberg .RA. “An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors”. *Nature Genetics,* 2008, 40(5): 499-507.

53. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO. “Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype”. *Clinical Cancer Research*, 2008, 14(5): 1368-1376
54. Gusterson B. “Do 'basal-like' breast cancers really exist?” *Nature Review Cancer,* 2009, 9(2): 128-34
55. Prat A., Parker J.S., Karginova O., Fan C., Livasy C., Herschkowitz J.I., He X., Perou C.M. “Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer”**.** *Breast Cancer Research*, 2010, 12:R68
56. Polyak K. “Breast cancer: origins and evolution”. *Journal Clinical Invest*, 2007, 117(11): 3155-63
57. Park S.Y., Lee H.E., Li H., Shipitsin M., Gelman R. and Polyak K. “Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer”. *Clinical Cancer Research* 2010, 16(3): 876-87.
58. Shackleton M., Quintana E., Fearon E.R. and Morrison S.J. “Heterogeneity in cancer: cancer stem cells versus clonal evolution”. *Cell* 2009, 138(5): 822-9.
59. Visvader J.E. and Lindeman G.J. “Cancer Stem Cells: Current Status and evolving complexities”. *Cell Stem Cell*, 2012, 10: 717-728
60. Reya T., Morrison S.J., Clarke M.F. and Weissman I.L. “Stem cells, cancer, and cancer stem cells”. *Nature,* 2001, 414(6859): 105-111
61. Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison S.J. and Clarke M.F. “Prospective identification of tumorigenic breast cancer cells”. *PNAS,* 2003, 100(7): 3983-3988
62. Singh S.K., Hawkins C., Clarke I.D., Squire J.A., Bayani J., Hide T., Henkelman R.M., Cusimano M.D. and Dirks PB. “Identification of human brain tumor initiating cells”. *Nature,* 2004, 432(7015): 396-401
63. Dalerba P., Dylla S.J., Park I.K., Liu R., Wang X., Cho R.W., Hoey T., Gurney A., Huang E.H., Simeone D.M., Shelton A.A., Parmiani G., Castelli C. and Clarke M.F. “Phenotypic characterization of human colorectal cancer stem cells”. *PNAS,* 2007, 104(24): 10158-10163
64. Siegel R., Naishadham D. and Jemal A. “Cancer statistics, 2012” *CA Cancer J Clin,* 2012, 62:10–29
65. La Vecchia C., Bosetti C., Lucchini F., Bertuccio P., Negri E., Boyle P. and Lev F. “Cancer mortality in Europe, 2000–2004, and an overview of trends since 1975”. *Annals of Oncology*, 2010, 21: 1323–1360
66. Weigelt B., Peterse J.L. and Van’t Veer L.J. “Breast cancer metastasis: markers and models”. *Nature Reviews Cancer*, 2005, 5: 591-602
67. Hanahan D. and Weinberg R.A. “Hallmarks of cancer: the next generation”. *Cell,* 2011, 144 (5): 646-674
68. Mehlen P. and Puisieux A. **“**Metastasis: a question of life or death”. *Nature Reviews Cancer* , 2006, 6: 449-458
69. Chambers A.F., Groom A.C. and MacDonald I.C. “Dissemination and growth of cancer cells in metastatic sites”. *Nature Reviews Cancer*, 2002, 2(8):563-72
70. Sahai E. “Illuminating the metastatic process”. *Nature Reviews Cancer*, 2007, 7(10):737-49
71. Cox E.A., Sastry S.K. and Huttenlocher A. “Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases”. *Molecular Biology Cell*, 2001, 12: 265–277

72. Ilina O., Bakker G.J., Vasaturo A., Hofmann R.M. and Friedl P. “Two photon laser-generated microtracks in 3D collagen lattices: principles of MMP-dependent and -independent collective cancer cell invasion”. *Phys. Biol*., 2011, 8: 015010
73. Wolf K., Wu Y.I., Liu Y., Geiger J., Tam E., Overall C., Stack M.S. and Friedl P. “Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion”. *Nature Cell Biology*, 2007, 9: 893–904
74. Hidalgo-Carcedo C., Hooper S., Chaudhry S.I., Williamson P., Harrington K., Leitinger B. and Sahai E. “Collective cell migration requires suppression of actomyosin at cell-cell contacts mediated by DDR1 and the cell polarity regulators Par3 and Par6”. *Nature Cell Biology*, 2011, 13: 49–58
75. Tambe D.T., Hardin C.C., Angelini T.E., Rajendran K., Park C.Y., Serra-Picamal X., Zhou E.H., Zaman M.H., Butler J.P., Weitz D.A..”Collective cell guidance by cooperative intercellular forces”. *Nat. Mater*. 2011, 10: 469–475
76. Allinen M., Beroukhim R., Cai L., Brennan C., Lahti-Domenici J., Huang H., Porter D., Hu M., Chin L., Richardson A. “Molecular characterization of the tumor microenvironment in breast cancer”. *Cancer Cell* 2004, 6:17–32
77. Friedl P. and Wolf K. “Tumor-cell invasion and migration: diversity and escape mechanisms”. *Nature Reviews*, 2003, 3:362-374
78. Netelenbos T., Zuijderduijn S., Van Den Born J., Kessler F.L., Zweegman S., Huijgens P.C. and Drager, A.M. “Proteoglycans guide SDF-1-induced migration of hematopoietic progenitor cells”. *Journal Leukoc. Biol.,* 2002, 72:353–362
79. Wyckoff J., Wang W., Lin E.Y., Wang Y., Pixley F., Stanley E.R., Graf T., Pollard J.W., Segall J. and Condeelis J. “A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors”. *Cancer Research*, 2004, 64: 7022–7029
80. Mu D., Cambier S., Fjellbirkeland L., Baron J.L., Munger J.S., Kawakatsu H., Sheppard D., Broaddus V.C. and Nishimura S.L. “The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1”. *Journal Cell Biology*, 2002, 157: 493–507
81. Bellahcéne A., Castronovo V., Ogbureke K.U., Fisher L.W. and Fedarko N.S. “Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer”. *Nature Review Cancer*, 2008, 8: 212–226
82. Thiery JP, Acloque H, Huang RY, Nieto MA. “Epithelial-mesenchymal transitions in development and disease”. *Cell*, 2009, 39:871–90
83. Nieto M.A. “The ins and outs of epithelial to mesenchymal transition in health and diseases”. *Ann Rev Cell Dev Biol* , 2011, 27:347-76
84. Kalluri R. and Weinberg R.A. “The basics of epithelial-mesenchymal transition”. 2009**,** 1420-1428
85. Thiery JP, Sleeman JP. “Complex networks orchestrate epithelial-mesenchymal transitions”. *Nature Review Molecular Cell Biology,* 2006, 7:131–42
86. Zeisberg M. and Neilson E.G. **“**Biomarkers for epithelial-mesenchymal transition”. *The Journal Clinical Investigation* , 2009,119(6): 1429-1437
87. Polyak K. and Weinberg R.A. “Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits”. *Nature Reviews Cancer*, 2009, 9: 265–73

88. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J. “Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis”. *Cancer Research*, 2011, 71:245–54
89. Yang M.H., Hsu D.S., Wang H.W., Wang H.J., Lan H.Y., Yang W.H., Huang C.H., Kao S.Y., Tzeng C.H., Tai S.K., Chang S.Y., Lee O.K., Wu K.J. “Bmi1 is essential in Twist1-induced epithelial mesenchymal transition”. *Nature Cell Biology,* 2010,12:982–92
90. Heuberger J. and Birchmeier W. “Interplay of Cadherin-mediated cell adhesion and canonical Wnt signaling.” *Cold Spring Harb Perspect Biol,* 2010, 2:a002915 1-24
91. Spaderna S., Schmalhofer O., Wahlbuhl M., Dimmler A., Bauer K. “The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer”. *Cancer Research,* 2008, 68:537-44
92. Whiteman E.L., Liu C.J., Fearon E.R. and Margolis B. “The transcription factor Snail represses Crumbs3 expression and disrupts apico-basal polarity complexes”. *Oncogene,* 2008, 27:3875-79
93. Kahlert C., Lahes S., Radhakrishnan P., Dutta S., Mogler C., Herpel E., Brand K., Steinert G., Schneider M., Mollenhauer M, Reissfelder C., Klupp F., Fritzmann J., Wunder C., Benner A., Kloor M., Huth C., Contin P., Ulrich A., Koch M. and Weitz J. “Overexpression of ZEB2 at the invasion front of colorectal cancer is an independent prognostic marker and regulates tumor invasion in vitro”. *Clinical Cancer Research*, 2011, 17 (24):1-10
94. Mani S.A., Guo W., Liao M.J., Eaton E.N., Ayyanan A. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell,* 2008, 133:704-15
95. Guo W., Keckesova Z., Liu Donaher J., Shibue T., Tischler V., Reinhardt F., Itzkovitz S., Noske A., Zurrer-Hardi U., Bell G., Tam W.L., Mani S.A., van Oudenaarden A. and Weinberg R.A. “Slug and Sox9 Cooperatively Determine the Mammary Stem Cell State”. *Cell*, 2012, 148, 1015–1028
96. Brabletz T., Jung A., Spaderna S., Hlubek F., Kirchner T. Opinion: migrating cancer stem cells: an integrated concept of malignant tumour progression. *Nature Reviews Cancer,* 20055:744–49
97. Li R., Liang J., Ni S., Zhou T. and Qing X. “A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts”. *Cell Stem Cell* 2009, 7:51–63
98. Bartel D.P. “MicroRNAs: genomics, biogenesis, mechanism, and function”. *Cell*, 2004, 116: 281–297
99. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. “Dicer is essential for mouse development”. *Nature Genetics,* 2003, 35(3):215-7.
100. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. “Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers”. *PNAS,* 2004, 101(9):2999-3004.
101. Lu J., Getz G., Miska E.A., Alvarez-Saavedra E., Lamb J., PecK D., Sweet-Cordero A., Ebert B.L., Mak R.H., Ferrando A.A., Downing J.R., Jacks T., Horvitz H.R. and Golub T.R. “MicroRNA expression profiles classify human cancers”. *Nature*, 2005, 435: 834–838

102. Calin G.A. and Croce C.M. “MicroRNA signatures in human cancers”. *Nature Reviews Cancer*, 2006, 6: 857–866
103. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. **“**A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia”. *N Engl Journal Medicine,* 2005, 353(17):1793-801.
104. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM. “MicroRNA gene expression deregulation in human breast cancer”. *Cancer Research,* 2005, 65(16):7065-70.
105. Ma L., Teruya-Feldstein J. and Weinberg R.A. “Tumour invasion and metastasis initiated by microRNA-10b in breast cancer”. *Nature*, 2007, 449: 682-689
106. Huang Q., Gumireddy K., Schrier M., le Sage C., Nagel R., Nair S., Egan D.A., Li A., Huang G., Klein-Szanto A.J., Gimotty P.A., Katsaros D., Coukos G., Zhang L., Puré E. and Agami R. ”The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis”. *Nature Cell Biology*, 2008, 10: 202 - 210
107. Valastyan S., Reinhardt F., Benaich N., Calogrias D., Szàsz A.M., Wang Z.C., Brock J.E., Richardson A.L. and Weinberg R.A. “A pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis”. *Cell*, 2009, 137 (6): 1032–1046
108. Brabletz S, Brabletz T. “The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer?” *EMBO Rep.* 201011:670–77
109. Brabletz S, Bajdak K, Meidhof S, Burk U, Niedermann G, et al.. The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. *EMBO Journal,* 2011, 30:770–82
110. Winter J., Jung S., Keller S., Gregory R.I. and Diederichs S. “Many roads to maturity: microRNA biogenesis pathways and their regulation”. *Nature Cell Biology,* 2009, 11: 228-234
111. Lee Y., Jeon K., Lee J.T., Kim S, Kim VN. “MicroRNA maturation: stepwise processing and subcellular localization”. *EMBO J.* 2002, 21(17):4663-70.
112. Köhler A. and Hurt E. “Exporting RNA from the nucleus to the cytoplasm”. Nature, 2007, 8:761-771
113. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim VN. “The nuclear RNase III Drosha initiates microRNA processing”. *Nature,* 2003, 425(6956):415-9.
114. Liu, J. et al. “Argonaute2 is the catalytic engine of mammalian RNAi”. *Science,* 2004, 305,1437-1441
115. Hutvágner G, Zamore PD. “A microRNA in a multiple-turnover RNAi enzyme complex”. *Science,* 2002, 297(5589):2056-60.
116. Reinhart B.J., Weinstein E.G., Rhoades MW, Bartel B., Bartel D.P. “MicroRNAs in plants”. *Genes Dev.* 2002, 16(13):1616-26.
117. Okamura K., Liu N. and Lai E. C. “Distinct mechanisms for microRNA strand selection by *Drosophila* Argonautes”. *Molecular Cell, 2009,* 36: 431–444
118. Dulbecco R, Bologna M and Unger M. “Differentiation of a rat mammary cell

line in vitro". *PNAS,* 1979, 76(3): 1256-60
119. Zucchi I., Sanzone S., Astigiano S., Pelucchi P., Scotti M., Valsecchi V., Barbieri O., Bertoli G., Albertini A., Reinbold R.A. and Dulbecco R. "The properties of a mammary gland cancer stem cell". *PNAS,* 2007, 104(25): 10476-81
120. Zucchi I. and Dulbecco R. "Proteomic dissection of dome formation in a mammary cell line". *Mammary Gland Biol Neoplasia*, 2002, 7(4):373-84
121. Zucchi I., Astigiano S., Bertalot G., Sanzone S., Cocola C., Pelucchi P., Bertoli G, Stehling M., Barbieri O., Albertini A., Schöler H.R., Neel B.G., Reinbold R.A. and Dulbecco R. "Distinct populations of tumor-initiating cells derived from a tumor generated by rat mammary cancer stem cells". *PNAS,* 2008, 105(44): 16940-5
122. Soriano J.V., Pepper M.S., Nakamura T., Orci L. and Montesano R. "Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells". *Journal Cell Science,* 1995, 108 (Pt 2): 413-30.
123. Silahtaroglu A.N., Nolting N., Dyrskjøt L., Berezikov E., Møller M., Tommerup N. and Kauppinen S. "Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification". *Nature Protocol*, 2007, 2(10): 2520-2528
124. Thisse B., Heyer V., Lux A., Alunni V., Degrave A., Seiliez I., Kirchner J., Parkhill J.P. and Thisse C. "Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening". *Methods Cell Biol*, 2004, 77:505-19
125. Tse J.C. and Kalluri R. "Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment". *Journal Cell Biochemistry*, 2007, 101(4):816-29
126. Li C.W., Xia W., Huo L., Lim S.O., Wu Y., Hsu J.L., Chao C.H., Yamaguchi H., Yang NK, Ding Q., Wang Y., Lai Y.J., LaBaff A.M., Wu T.J., Lin B.R., Yang M.H., Hortobagyi G.N. and Hung M.C. "Epithelial-mesenchymal transition induced by TNF-α requires NF-κB-mediated transcriptional upregulation of Twist1". *Cancer Research,* 2012, 72(5):1290-300
127. Scheel C., Eaton E., Hsin-Jung Li S., Chaffer C.L., Reinhardt F., Kah K.J., Bell J., Guo W., Rubin J., Richardson A.L. and Weinberg R.A. "Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast". *Cell*, 2011, 145: 926–940
128. Siegel P.M. and Massegué J. "Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer" *Nature Reviews Cancer* , 2003, 3: 807-820
129. Massague J. "TGFβ in cancer". *Cell,* 2008, 134:215–30
130. Giampieri S., Manning C., Hooper S., Jones L., Hill C.S. and Sahai E. "Localized and reversible TGFβ signalling switches breast cancer cells from cohesive to single cell motility". *Nature Cell Biology,* 2009, 11:1287–96
131. Lee Y.B., Bantounas I., Do-Young Lee D.Y., Phylactou L., Caldwell M.A. and Uney J.B. "Twist-1 regulates the miR-199a/214 cluster during development" *Nucleic Acids Research*, 2009, 37(1): 123–128
132. Yin G., Chen R., Alvero A.B., Fu H.H., Holmberg J., Glackin C., Rutherford T. and Mor G. "TWISTing stemness, inflammation and proliferation of epithelial ovarian cancer cells through MIR199A2/214". *Oncogene*, 2010, 1–9

133. Valsesia-Wittmann S, Magdeleine M, Dupasquier S, Garin E, Jallas A.C., Combaret V,Krause A., Leisser P. and Puisieux A. “Oncogenic cooperation between HTwist and N-Myc overrides failsafe programs in cancer cells”. *Cancer Cell*, 2004, 6: 625–630.
134. Entz-Werle N, Stoetzel C, Berard-Marec P, Kalifa C, Brugiere L, Pacquement H Schmitt C., Tabone M.D., Gentet J.C., Quillet R., Oudet P., Lutz P., Babin-Boilletot A., Gaub M.P. and Perrin-Schmitt F. “Frequent genomic abnormalities at TWIST in human pediatric osteosarcomas”. *Int J Cancer*, 2005, 117:349–355.
135. Yang J., Mani S.A., Donaher J.L., Ramaswamy S., Itzykson R.A, Come C., Savagner P., Gitelman I., Richardson A. and Weinberg R.A. “Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis”. *Cell*, 2004, 117: 927–939
136. Mironchik Y, Winnard Jr PT, Vesuna F, Kato Y, Wildes F, Pathak AP., Kominsky S., Artemov D., Bhuiwalla Z., Van Diest P., Burger H., Glackin C. and Raman V. “Twist overexpression induces in vivo angiogenesis and correlates with chromosomal instability in breast cancer”. *Cancer Research*, 2005, 65: 10801
137. Cheng G.Z., Zhang W. and Wang LH. “Regulation of cancer cell survival, migration, and invasion by Twist: AKT2 comes to interplay”. Cancer Res (2008b), 68: 957–960
138. Gal A., Sjöblom T., Fedorova L., Imreh S., Beug H. and Moustakas A. “Sustained TGFβ exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis”. *Oncogene*, 2008, 27: 1218–1230
139. Cheng GZ, Zhang WZ, Sun M, Wang Q, Coppola D, Mansour M. et al.. “Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function”. *J Biol Chem,* 2008c, 283:14665–14673.
140. Kolsch V, Seher T, Fernandez-Ballester GJ, Serrano L, Leptin M.. “Control of Drosophila gastrulation by apical localization of adherens junctions and RhoGEF2.” Science, 2007, 315:384–86
141. Martin A.C., Kashube M. and Wieschaus E.F. “Pulsed actin-myosin network contractions drive apical constriction”. 2009, Nature, 22 (7228): 457-495
142. Dunah A.W., Hueske E., Wyszynski M., Hoogenraad C.C., Jaworski J., Pak D.T., Simonetta A., Liu G. and Sheng M. “LAR receptor protein tyrosine phosphatases in the development and maintenance of excitatory synapses”. *Nature Neuroscience*, 2005, 4: 458-467
143. Müller T., Choidas A., Reichmann E., Ullrich A. “Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration”. *The Journal of Biological Chemistry* , 1999, 274(15):10173-10183
144. Beltran P.J. and Bixby J.L. “Receptor protein tyrosine phosphatases as mediators of cellular adhesion”. *Front. Bioscience*, 2003, 8: D87–D99
145. Debant A., Serra-Pages C., Seipel K., O'Brien S., Tang M., Parks S.H. and Streuli M. “The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains”. *PNAS*, 1996, 93: 5466-5471

146. Medley Q.G., Buchbinder E.G., Tachibana K., Ngo H., Serra-Pages C. and Streuli M. “Signaling between Focal Adhesion Kinase and Trio”. *Journal of Biological Chemistry*, 2003, 278(15): 13265-13270
147. Serra-Pages C., Kedersha N.L., Fazikas L., Medley Q., Debant A. and Streuli M. “The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions”. *Embo Journal*, 1995, 14:2827-38.
148. Serra-Pages C., Streuli M. and Medley Q.G. “Liprin phosphorylation regulates binding to LAR: evidence for Liprin autophosphorylation”. *Biochemistry*, 2005, 44(48): 15715–15724.
149. Billottet C., Elkhatib N., Thiery J.P. and Jouanneau J. “Targets of Fibroblast Growth Factor 1 (FGF-1) and FGF-2 signaling involved in the invasive and tumorigenic behavior of carcinoma cells”. *Molecular Biology of the Cell*, 2004, 15: 4725–4734
150. Kypta R.M., Su H. and Reichard L.F. “Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex”. *Journal Cell Biology*, 1996, 134:1519–1529
151. Bonvini P., An W.G., Rosolen A., Nguyen P., Trepel J., Garcia de Herreros A., Dunach M. and Neckers L.M. “Geldanamycin abrogates ErbB2 association with proteasome-resistant beta-catenin in melanoma cells, increases beta-catenin-E-cadherin association, and decreases beta-catenin-sensitive transcription”. *Cancer Research,* 2001, 61(4):1671-7
152. Lilien J. and Balsamo J. “The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin”. *Current Opinion Biology*, 2005, 17(5):459-65
153. Leadem Daugherty R. and Gottardi C.J. “Phospho-regulation of β-Catenin adhesion and signaling functions”. *Physiology*, 2007, 22: 303-309
154. Xu Y. and Fisher G.J. “Receptor type protein tyrosine phosphatases (RPTPs): roles in signal transduction and human disease”. *Journal Cell Communication Signalling*, 2012, 6(3): 125–138
155. Östman A., Hellberg C. and Böhmer F.D. “Protein-tyrosine phosphatases and cancer”. *Nature Reviews Cancer*, 2006, 6: 307-320
156. Wang Z., Shen D., Parsons D.W., Bardelli A., Sager J., Szabo S., Ptak J., Silliman N., Peters B.A., van der Heijden M.S., Parmigiani G., Yan H., Wang T.L., Riggins G., Powell S.M., Willson J.K., Markowitz S., Kinzler K.W., Vogelstein B. and Velculescu V.E. “Mutational analysis of the tyrosine phosphatome in colorectal cancers”. *Science*, 2004, 304: 1164–1166
157. DiMeo T.A., Anderson T., Phadke P., Feng C., Perou C.M., Naber S. and Kuperwasser C. “A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and Epithelial-Mesenchymal Transition in basal-like breast cancer”. *Cancer Research*, 2009, 69:5364-5373
158. Kowalski P.J., Rubin M.A. and Kleer C.G. “E-cadherin expression in primary carcinomas of the breast and its distant metastases”. *Breast Cancer Research*, 2003, 5:R217-R222
159. Chao Y., Shepard C.R. and Wells A. “Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition”. *Mol Cancer*, 2010, 9: 179

160. Wendt M.K., Taylor M.A., Schiemann B.J. and Schiemann W.P. “Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer” *Molecular Biology Cell*, 2011, 22(14): 2423-35
161. Pencheva N.,Tran H., Buss C., Huh D., Drobnjak M., Busam K. and Tavazoie S.F. “Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis”. *Cell*, 2012, 151: 1–15
162. Marchini S., Cavalieri D., Fruscio F., Calura E., Garavaglia D., Fuso Nerini I., Mangioni C., Cattoretti G., Clivio L., Beltrame L., Katsaros D., Scarampi L., Menato G., Perego P., Chiorino G., Buda A., Romualdi C. and D’Incalci M. “Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour tissue collections”. *Lancet Oncology*, 2011, 1-12
163. Song G., Zeng H., Li J., Xiao L., He Y., Tang Y. and Li Y. “MiR-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer”. *Biol. Pharm. Bull.*, 2010, 33:1822–1827
164. Brenner B., Hoshen M.B., Purim O., David M.B., Ashkenazi K., Marshak G., Kundel Y., Brenner R., Morgenstern S., Halpern M., Rosenfeld N., Chajut A., Niv Y. and Kushnir M. “Micrornas as a potential prognostic factor in gastric cancer”. *World J. Gastroenterol.*, 2011, 17:3976–3985.
165. Ueda T., Volinia S., Okumura H., Shimizu M., Taccioli C., Rossi S., Alder H., Liu C.G., Oue N., Yasui W., Yoshida K., Sasaki H., Nomura S., Seto Y, Kaminishi M., Calin G.A. and Croce C.M. “Relation between microrna expression and progression and prognosis of gastric cancer: A microrna expression analysis”. *Lancet Oncology*, 2010, 11:136–146

ACKNOWLEDGEMENTS

I would like to express my sincere and massive gratitude to Dr Ileana Zucchi who believed in me giving me the opportunity to conduct this research project. I am grateful for her constant help and scientific guide.

I deeply thank all the members of my lab for their scientific and personal support, particularly Dr Reinbold for the critic reviewing of my PhD work.

I respectfully thank the Institute of Biomedical Tecnologies of the National Research Council and the Director Dr Luigi Zecca.

I thank my tutors Prof Mario (Mago) Clerici and Prof Maria Luisa Villa and all the members of the Molecular Medicine PhD School of the University of Milan.

I thank Dr Simonetta Astigiano and collaborators from IRCCS-AOU San Martino of Genova and Dr Davood Sabour and collaborators from Max Plank Institute for Molecular Biomedicine, Cell and Developmental Biology of Muenster for the great *in vivo* works.

I thank Dr Giovanni Bertalot and collaborators of the Azienda Ospedaliera di Manerbio e Desenzano for the precious histopathology work.

Finally I would like to express my greatest gratitude to my family and to my supersweet nephews Andrea and Leonardo.