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Abstract

A great hallmark of breast cancer is the absence or presence of estrogen receptors ERα and ERβ, with a dominant role in cell proliferation, differentiation and cancer progression. Both receptors are related with Epithelial-to-Mesenchymal Transition (EMT) since there is a relation between ERs and extracellular matrix (ECM) macromolecules expression, and therefore, cell-cell and cell-ECM interactions. The endocrine resistance of ERα endows epithelial cells with increased aggressiveness and induces cell proliferation, resulting into a mesenchymal phenotype and an EMT status. ERα signaling may affect the transcriptional factors which govern EMT. Knockdown or silencing of ERα and ERβ in MCF-7 and MDA-MB-231 breast cancer cells respectively, provoked pivotal changes in phenotype, cellular functions, mRNA and protein levels of EMT markers, and consequently the EMT status. Mesenchymal cells owe their migratory and invasive properties to invadopodia, while in epithelial cells, lamellipodia and filopodia are mostly observed. Invadopodia, are actin-rich protrusions of plasma membrane, promoting proteolytic degradation of ECM and tumor invasion. Cortactin and MMP-14 govern the formation and principal functions of invadopodia. *In vitro* experiments proved that lumican inhibits cortactin and MMP-14 expression, alters the formation of lamellipodia and transforms mesenchymal cells into epithelial-like. Conclusively, lumican may inhibit or even reverse the several metastatic features that EMT endows in breast cancer cells. Therefore, a lumican-based anti-cancer therapy which will pharmacologically target and inhibit EMT might be interesting to be developed.

Keywords

EMT; invadopodia; breast cancer; lumican

1) Cell migration and invadopodia

Cell migration is a mechanical physiological process occurring during embryogenic morphogenesis, bone homeostasis, tissue repair and regeneration. Cell invasion consisting in the breaching of tissue barriers like endothelial basement membrane is a basic function of immune cells to respond and to prevent infections. Invasion takes place during disease progression such as cancer invasion of extracellular matrix (ECM) and final metastasis [1].
There are distinct types of cell migration: cells can migrate as individual cells or collectively in group of cells moving together, just retained by intercellular interactions. Cells can move in two different mechanical ways referred to “amoeboid” or “mesenchymal” movements [2, 3]. In general, amoeboid movement is protease-independent, but requires pores or channels (more than 3-5 μm in diameter) for cells to squeeze through, whereas mesenchymal movement is protease-dependent and is necessary to traverse nanoporous matrices such as basement membranes [4, 5].

Cells migrating by an amoeboid movement move at high speeds (4 mm/min) gliding on the substratum and developing relatively weak adhesion and traction [1, 2, 6], whereas cells migrating individually by a mesenchymal mode dynamically form cytoplasmic protrusions and adhesions to the microenvironment, perform cell body translocation, release of adhesions and detachment of the cell’s rear which regulate speed and directional persistence [3, 7-11]. All cells migrating by mesenchymal movement are more aggressive to surrounding tissues as they can widen spaces and gaps by degrading ECM components through an extracellular proteolysis [2]. Cell migration needs dynamic interactions between the migrating cells and the surrounding microenvironment. In particular, dynamic attachment, traction and detachment of cells or cell protrusions to the substratum allow cell movement. In experimental studies cell movement is induced by nutrients which attract cells in two different ways according to the different environment: a two-dimensional (2D) locomotion by which cells can move only in two directions, and a three-dimensional (3D) movement by which cells are able to move or interact with the microenvironment structure in all the three spatial dimensions. In both types of migration the cells have to acquire a motile phenotype by developing some plasma membrane protrusions through an actin cytoskeleton remodeling and formation of actin-based structures which allow the cells to adhere to as well to probe and sense different molecules of ECM [12-14]. Actin filaments form the cytoskeleton in mammalian cells and undergo constant remodeling during cell migration [15]. In particular, new actin filaments are continuously synthetized at the leading edge of migrating cells to form new plasma membrane protrusions which promote and drive cell migration direction [12, 16]. Cell adhesion to the ECM is ensured by an integrin-actin linkage system, where integrins are the major and best-characterized transmembrane receptors which favor dynamic interactions between ECM and actin cytoskeleton during cell movement [3].

The 2D locomotion is characterized by the formation of cytoplasmic protrusions showing different shapes: filopodia (needle shape), pseudopodia (round), lobopodia (cylindrical) and lamellipodia (flat veils) [17]. Uropods which have many more “folds” or “ridges”, magnupodia (thick and very long protrusions extending more than 330 μm away from the cell) and tenupodia (very thin straight processes often connecting distant cells) were also described [18, 19]. The most common cytoplasmic protrusions for cell migration are filopodia and lamellipodia; the firsts are thin, finger-like cytoplasmic projections formed by parallel bunches of actin filaments, whereas the second ones look like flat broad membranous protrusions located at the leading edge of the migrating cells [20, 21].

In 3D movement, cells develop specialized types of integrin-mediated adhesions through plasma membrane protrusions named podosomes and invadopodia, collectively known as invadosomes, which establish a close contact with the ECM and are able to invade dense connective tissues by performing a proteolytic matrix degradation [14, 22, 23]. Tumor cells could activate invadosomes to firstly degrade the basement membrane surrounding the primary tumor, then to invade the connective tissue and penetrate into the lymphatic or blood vessels (intravasation phase). Cancer cell nucleus has a limited deformability so that the ECM proteolytic degradation seems to be necessary mainly to enlarge the pores in the matrix, allowing the nucleus migration [24].

The role of invadopodia in proteolytic degradation of vascular basement membranes seems to be essential also during intravasation and metastasis [25, 26]. Invadopodia could play other important roles such as orienting tumor cells toward chemotactic signals as they are also involved in chemotaxis during migration in both 2 and 3D [27].

Podosomes are actin structures connecting the cytoskeleton with the plasma membrane and appear like small plasma membrane protrusions distributed at the cell surface, measuring only 0.4 μm in length and 1 μm in diameter and undergoing constant disassembly and reformation [28, 29]. They are associated with normal cells, such as macrophages, osteoclasts, dendritic cells, epithelial cells, smooth muscle cells and fibroblasts and consist in a branched actin core containing proteins involved in actin polymerization and a surrounding ring rich in β2 and β3 integrin receptors and adhesion proteins such as talin, vinculin and paxillin [3, 4, 30, 31]. Podosomes act like probing or palpating organelles well distributed on cell membrane of the myelomonocytic lineage, such as macrophages [32], immature dendritic cells [33] and osteoclasts [34]. They are mainly related to cell migration and diapedesis of blood leukocytes even though they may assume an invasive function in facilitating the tumor cell invasion process by a proteolytic activity [22, 29, 35, 36].

Invadopodia, firstly described in melanoma and invasive breast cancer cells [37, 38], are finger-like protrusions developing from the ventral side of the invading cells and correspond

to specialized tightly-packed, organelle-free, actin-rich protrusions of the plasma membrane structures as actin polymerization is crucial for the formation and function of invadopodia. They are morphologically similar to podosomes but they appear larger than podosomes with a length which may reach 2-5 μm and a diameter up to 8 μm [39], even though other authors reported smaller size because invadopodia can cluster together [40]. Moreover, cells are able to form numerous podosomes (more than 100) but only few invadopodia (between one and ten) [39]. Furthermore, podosomes have a lifetime of several minutes whereas invadopodia can persist for over 1 hour [41].

Differently to podosomes, invadopodia lack a ring structure as observed at the transmission electron microscope, the actin filaments appear throughout the core of the invadopodial protrusion, excluding other cytoplasmic structures from the core area [42]. Both podosomes and invadopodia are constituted by a dense filamentous (F)-actin core containing actin-regulating proteins, with polymerization activators and, filament crosslinkers, nucleators and binders, surrounded by proteins involved in regulation, adhesion and scaffolding, including integrins, kinases, GTPases, and adaptor proteins. Major molecules of invadosomes include the scaffold protein Tks5, the actin regulators cortactin, Wiskott-Aldrich syndrome protein family members (WASP or N-WASP) and cofilin, and MMP-14 [43].

2) Relation of invadopodia with cancer

Invadopodia are described in invading cells, such as metastatic tumor cells [39, 44]. In fact, they are always closely located to the ECM and represent enzymatically matrix-degrading structures burrowing across tissue barriers through the release of matrix metalloproteases (MMPs) which mainly include MMP-14, MMP-2 and MMP-9 [12, 16, 39, 40]. These MMPs, and in particular MMP-14, are secreted to the site of invadopodia adhesion through vesicles [45]. Even though the main function of invadopodia in tumors is to promote the proteolytic matrix degradation to favor cancer cell invasion, other functions have been suggested: the proteolytic action of these cytoplasmic protrusions to create space in the ECM could favor tumors growth or alternatively the pericellular proteolytic activity at invadopodia surface could activate various growth factors to drive tumor growth [43] and favor angiogenesis [46, 47].

Both podosomes and invadopodia can degrade the ECM and increase the ability of cells to cross tissue barriers, but ECM degradation is deeper and more focused when promoted by invadopodia [39]. Podosomes usually degrade the exposed surface of the matrix, whereas invadopodia, are able to penetrate deeper among the ECM components [42]. Invadopodia have to be considered high dynamic structures which support adhesion-secretion-movement: after a stimulus, they first assemble interaction between cell and ECM components, such as fibronectin or vitronectin molecules, then they release MMPs degrading the matrix and finally disassemble again, allowing cell movement [48].

One major protein which is required for the assembly of invadopodia by regulating the F-actin-enriched invadopodial cytoskeleton is cortactin, a substrate of Src family tyrosine kinases. By promoting invadopodia formation, function and disassembly, it is related to cancer cell migration, invasion, intravasation, extravasation and metastasis [49-53]. An overexpression of cortactin is frequently reported in several types of invasive cancers so that it is widely used as a marker of invadosomes [48]. Similarly, inhibition of cortactin leads to the inhibition of invadopodia formation and reduces metastasis to distant organs [54, 55].

Different morphologic conformations of invadosomes such as aggregates, individual dots, rosettes or linear shaped structures along type I collagen fibrils can be observed in the same cell, thus supporting the concept of invadosome plasticity [22]. The relationship between podosomes and invadopodia is not clearly defined in the literature: it was also suggested that podosomes and invadopodia may share a common primordial precursor able to adapt to its microenvironment and modulate its morphology in relation to the different substrates [39, 44, 56, 57]. Moreover, a close distinction in function between pseudopodia and invadosomes seems to be discussed: some authors suggested that a high degree of molecular integration and cross talk between pseudopodia and invadopodia allowing efficient invasion coupled migration in both 2 and 3D may be also possible [27, 58]. Formation of both invasive protrusions (invadopodia) and locomotory protrusions (pseudopodia) is regulated by stromal cells in the tumor microenvironment like fibroblasts in squamous cell carcinoma [59] and macrophages in breast carcinoma [60, 61]. Moreover, ECM rigidity seems to influence the formation of invadopodia so that alterations in matrix stiffness may be related to cancer disease and progression [61]. It is worth noticing that dense fibrillar collagen so as transient mechanical strains promote the maturation of invadopodia and enhances cancer cell invasion *in vitro* [40, 42]. In particular, it was demonstrated that fibrillar collagen I is a physiological inducer of a novel class of invadosomes called “linear invadosomes” which might act as collagen I fibril sensors and are able to remodel the ECM [62]. In addition, hypoxia of peritumoral stroma can promote mesenchymal invasion in breast cancer cells through the upregulation of structural components of the actin cytoskeleton machinery involved in invadopodium formation [63].

3) Lumican, a Class II Small Leucine Rich Proteoglycan, as a regulator of Epithelial-to-Mesenchymal Transition

ECM is a three-dimensional network of macromolecules which provides structural and biochemical support to the surrounding cells. ECM also regulates intracellular communication and affects cell behavior. Through the fine-tuned interactions between cell surface receptors and ECM components, gene expression and diverse functional properties are affected. ECM remodeling can occur in both physiological and pathological conditions [64].

Proteoglycans (PGs) are key components of the ECM. PGs are constituted by a core protein to which linear and highly anionic chains of glycosaminoglycans (GAGs) are covalently attached. PGs are so called as multifunctional key effectors, since they are involved in a plethora of pathophysiological processes, such as cancer [65-67]. PGs expression is remarkably altered during tumor development and growth and their remodeling on the tumor ECM and cell membranes influences major cancer cell properties, such as cell proliferation, migration, invasion, angiogenesis and adhesion [68]. The most abundantly expressed PGs in ECM are the Small Leucine-Rich PGs (SLRPs). Their organization is pericellular and their core protein substituted by negatively charged GAG chains enable the interactions of SLRPs with matrix effectors, such as cytokines, growth factors and cell surface receptors. These interactions lead to the regulation of crucial cell functional features, i.e.: migration, autophagy, angiogenesis and metastatic potential of cancer cells [69-71]. SLRPs comprise of 18 members classified into five classes according to the conservation and homology at the protein and genomic levels. Lumican is a Class II SLRP, characterized by high molecular heterogeneity according to the tissue due to its glycosylation. Human lumican core protein is a protein of338 amino acids, including an 18 amino acid signal peptide and three major domains, a negatively charged N-terminal domain containing cysteine and sulfated tyrosine residues, a central part containing 9 Leucine Rich Repeats (LRR), a terminal domain of 66 amino acids containing 2 conserved cysteine and 2 LRRs. The structure of lumican is illustrated in Figure 1.

Lumican expression is abundant in the ECM of many tissues, such as skin, kidney, breast, colon, pancreas, cartilage, etc. [72]. In certain tissues, lumican is linked positively with tumor aggressiveness, while in some other tissues, negatively [73]. In breast carcinoma, as well as in melanoma, lumican expression is increased in the stroma [74-78]. Increased expression of lumican in the stroma of breast carcinomas is not a prognostic factor for breast cancer. However, enhanced lumican expression is related with increased levels of metastasis, decreased levels of estrogen receptors (ERs) expression and the young age of the patients. On the other hand, the decreased lumican expression is reported to be related with poor clinical outcome and survival of the patient [76, 78].

Several published studies refer to the anti-cancer action of lumican, although the anti-tumorigenic mechanism behind it, is still not totally clarified. Data from our research group have suggested an anti-cancer effect of lumican in melanoma. *In vitro*, the core protein of lumican (37 kDa) was reported to increase melanoma cell adhesion [79], and its glycosylated form (57 kDa) inhibited cell migration and invasion, with simultaneous changes in the actin network and focal adhesion complexes [80-83]. At *in vivo* level, when syngeneic mice were injected with B16F1 melanoma cells stably transfected with lumican expressing plasmid (Lum-B16F1), the size and the number of the lung metastatic nodules were significantly decreased in comparison with the Mock-B16F1 cells [84]. Angiogenesis was also altered, as the number of the blood vessels in the metastatic nodules was decreased [85].

Interaction of lumican with ECM key components mediated by membrane receptors or MMP-14, renders lumican a possible anticancer effector [73, 86]. Lumican (57 kDa) decreased the migration of endothelial cells, by inhibiting the expression and the activity of MMP-9 and MMP-14 through the interaction with integrins [87]. Several cell functions affected by lumican, are integrin mediated. Interaction of lumican with $\alpha 2\beta 1$ integrin affects the phosphorylation of FAK and alters the actin network [81]. The interaction of lumican with integrins was also reported in osteosarcoma, as lumican had an impact on osteosarcoma cell adhesion *via* inhibition of TGFβ-2. The altered expression and activity of TGFβ-2 triggered downstream modification of the signaling cascade of pSmad2, enhanced the activity of $\beta 1$ integrin with simultaneous decreased FAK phosphorylation. It is not clarified yet if TGF-β is a mediator of Epithelial-to-Mesenchymal Transition (EMT)/ Mesenchymal-to-Epithelial Transition (MET), in synergy with lumican. MMP-14 is another crucial mediator of the anti-tumorigenic mechanism of lumican, as it plays pivotal role in cell migration, invasion and angiogenesis through the activation of downstream MMPs, as well as through regulation of the activation of migration-related molecules, such as integrins and several signaling pathways [88, 89].

EMT is an important biological mechanism of normal development, where normal, epithelial cells undergo a plethora of biochemical changes and end up with altered phenotype and altered cellular functional properties. More specifically, epithelial cells are transformed into mesenchymal, spindle-like shaped cells with enhanced cell migration, as well as invasive and metastatic properties in the case of malignancies. There are some specific molecular events that trigger the initiation of EMT, such as activation of transcription factors, re-arrangement of cytoskeletal proteins and altered expression of microRNAs. MET is the reverse procedure of EMT, where mesenchymal cells are transformed into an epithelial status [90].

It was recently reported that the expression of estrogen receptors ERα and ERβ of breast cancer cells is related with EMT. Breast cancer is the most common type of cancer among women and a great hallmark of breast cancer is the presence or absence of estrogen receptors (ERα and ERβ). ERα is extensively studied, since it is the major ER subtype in the mammary epithelium, so it can serve as a prognostic marker, too. Moreover, 70% of breast cancer cases are featured as ERα (+), rendering the 17β-estradiol (E2)/ERα signaling as very important. ERα provides cells with enhanced migratory and invasive capacities, which eventually turns the epithelial cells into mesenchymal, triggering an EMT status [91]. This may be explained by the fact that most transcriptional factors implicated in EMT, are affected by ERα signaling [92, 93]. Bouris *et al.*, reported that the knockdown of ERα in the low-invasive, epithelial MCF-7 cells, provokes a potent EMT status, as well as significant alterations in the gene expression of several macromolecules of the ECM and cell functional properties of breast cancer. The silencing of ERα was achieved using shRNA lentiviral particles, triggering an altered phenotype, enhanced cell proliferation, migration and aggressiveness, tremendous decrease of the gene expression of the epithelial marker E-

cadherin and simultaneously great enhancement of the gene expression of mesenchymal markers, such as vimentin, and slug/SNAIL-2.

The addition of lumican in MCF-7 cells, either control or the ERα silenced, affected the receptor-associated functional features of breast cancer, the gene expression of matrix macromolecules, as well as the EMT status. Concerning MCF-7 cells, cell morphology was affected, since lumican rendered MCF-7 cells as more epithelial. Lumican triggered cell accumulation, as well as a more globular phenotype. The number of cell-cell junctions has increased, and the cells are in tight contact. Lumican endowed MCF-7 cells with a more grouped, ovoid and more flattened shape, as compared to the untreated cells. The gene expression of EMT markers was notably affected, as the epithelial marker E-cadherin was increased, while vimentin was further decreased. The effect of lumican was also profound in the cell functional properties, such as migration and invasion, as well as the expression profile of crucial matrix effectors in MCF-7 cells. Lumican suppressed cell migration, which comes in agreement with the observations in cell morphology. Cell invasion was also suppressed, although MCF-7 cells exert low invasive dynamic [94].

Although the biological role of ERα is extensively studied, the biological role of ERβ is not clearly elucidated. To examine it, Piperigkou *et al.* suppressed its expression using a shRNA against human ERβ, through which a suppression of ERβ mRNA by 70% was achieved [95]. The MDA-MB-231 cells that had undergone ERβ suppression, tend to gain a more epithelial phenotype and more cell-cell adhesion junctions, leading to a potent MET state. Cell morphology and especially epithelial-to-mesenchymal transition has been well correlated with the high invasive potential of breast cancer cell [91, 95, 96]. Gene expression of EMT markers was affected, with significantly enhanced levels of E-cadherin, as well as decreased levels of vimentin, zeb-1, slug/SNAIL-2 and fibronectin. Cell functional properties were also affected, associated with the alteration MMPs and TIMPs alteration of expression and activity.

Upon treatment with lumican, crucial alterations were observed in cell morphology of MDA-MB-231. Lumican-treated MDA-MB-231 cells exhibit a significant increase in the I/L ratio and cell-cell contacts, in agreement with the significant inhibitory effect of lumican in the invasion of MDA-MB-231 cells. The cell populations display heterogeneity, as flattened and spindle-like cells co-exist and the number of cell-cell junctions seems to be increased. Similarly, shERβMDA-MB-231 cells upon treatment with lumican, exerted a more epithelial phenotype, as their shape was observed as wider, ovoid and flattened, with increased I/L ratio and rare cytoplasmic protrusions like lamellipodia and filopodia [97].

Lumican treatment endowed significant downregulation in the gene expression of mesenchymal markers, such as slug/snail-2, zeb-1, vimentin and fibronectin. The gene expression of the epithelial marker E-cadherin was tremendously increased in the highly

invasive MDA-MB-231 cell line, while slightly increased in the shERβMDA-MB-231, rendering lumican a potent regulator of EMT. Cell functional properties, such as proliferation, migration and invasion were downregulated upon lumican treatment, and in most assays in an ERβ dependent mode. It is worth noticing that lumican significantly inhibited both the proteolytic activity levels of MMP-14 and as well the gene expression levels of MMP-7, coming in agreement with the observed effect of lumican in cell functional properties. Conclusively, lumican altered cell morphology, including cell-cell junctions and provoked EMT/MET reprogramming. These data underline the anticancer effect of lumican, related to the ER status and could be potentially applied for designing novel pharmaceutical agents for breast cancer therapy [97].

4) Lumican as an effector on invadopodia formation

Taking into consideration the anti-migratory and anti-invasive effects of lumican, as well as the fact that the invadopodia are formed at the leading edge of cancer cells, enabling their migration and invasion, it was of great importance to investigate the lumican effect on invadopodia formation. It was reported that when melanoma cells were grown onto lumican coating, cell morphology was altered and localization of actin filaments was re-arranged [82]. Coulson-Thomas *et al.* seeded bone derived prostate cancer cells upon lumican coating, which provoked modification of the elongated shape of the cells into more rounded, as well as reduction of the number of the cellular protrusions, such as lamellipodia and invadopodia [98]. Apart from the actin filaments, intermediate filaments, such as keratin cytoskeleton, play pivotal role in the process of cell migration.

Keratin 8 and 18 are expressed separately in normal keratinocytes, however they are found to be co-expressed in planocellular cancer cells, in the form of 8/18 filaments. This co-expression of keratin 8/18 filaments enables the invasion of normal keratinocytes in the basement membrane [99]. It is already reported that keratin 8 re-organization provokes increased levels of cell motility, high levels of keratin 8 and 18 are signals of metastatic progression and poor clinical outcome in squamous cell carcinomas (SCCs) [100-102]. On the other hand, decreased expression of keratin 8 and keratin 18 have been correlated with low α6β4 integrin expression, cancer cell migration and invasion [103]. When Coulson-Thomas *et al.*, seeded prostate cancer cells upon lumican coating, both keratin 8 and 18 expression was decreased, as well as their organization from cellular protrusions was modified into perinuclear localization [98]. In addition, upon lumican treatment, de-stabilization of focal adhesion complexes was observed to lead to inhibition of cell migration. More specifically, Zona occludens protein 1 (ZO-1), a protein which plays pivotal role in cell migration, since it is localized in lamellipodia and in intracellular tight junctions, presented a decreased expression and was concentrated in the cell membrane and not in the cellular protrusions [104].

Based on the anticancer effect of lumican that effectively regulates ERs-associated functional properties of breast cancer cells, expression of matrix macromolecules and EMT,

the analysis of lumican effects in cell morphology and invadopodia formation of invading breast cancer cells was performed by our group (Karamanou *et al.*, submitted to FEBS Journal [105]). The effects of lumican were evaluated in three breast cancer cell lines, the highly metastatic ERβ-positive MDA-MB-231, the respective ERβ-suppressed (shERβMDA-MB-231) and the low invasive ERα-positive MCF-7 breast cancer cells. We investigated cell morphological aspects of invading cells in various matrices by scanning electron microscopy. The obtained data suggested that the expression of invadopodia marker (cortactin), focal adhesion proteins (vinculin, talin), hyaluronan and its receptor variants and biosynthetic enzymes (CD44, CD44s, CD44-v2, -v3, -v8, HAS-1, -2, -3) were altered by the presence of lumican in association with the level of expression of ERs. The expression of CD44 and CD44s was high in MDA-MB-231 cells, moderate in shERβMDA-MB-231 and significantly decreased in the low-metastatic MCF-7 cells, underlying a correlation of CD44 with the ER status.

Morphological changes that cells undergo after invasion were evaluated after seeding onto different matrix-coated surfaces. Cells were cultured for both 24 and 48 hours and seeded on the upper surface of a Millipore filter coated with collagen type I and were observed by Scanning Electron Microscopy (SEM) (Figure 2). Regarding MDA-MB-231 breast cancer cells, SEM analysis revealed their elongated, spindle-like shape, as well as their numerous cytoplasmic protrusions. The cellular surface of MDA-MB-231 was irregular and non-smooth. Upon 100nM lumican treatment in serum-free conditions for 48h, MDA-MB-231 breast cancer cells seemed more flattened with a smoother surface and a decreased number of invadopodia, as seen in Figure 1. Similarly, 48h lumican treatment evoked morphological changes in MCF-7 breast cancer cells during invasion in different collagen substrates. SEM analysis revealed the flattened and globular morphology of MCF-7 cells, as well as a diminished number of invadopodia and even few microvilli. After 24h of treatment with 100nM lumican, SEM revealed morphological alterations in MCF-7, as they appeared as more grouped more globularly-shaped. The cytoplasmic membrane was observed as smooth with absence of microvilli, microvesicles and invadosomes. Exosomes were also observed (Karamanou *et al.*, submitted to FEBS J, [105]).

As already referred, invadopodia are membrane protrusions of invasive cancer cells, involved in the focal pericellular degradation of ECM. It is very critical to evaluate the three breast cancer cell lines of different estrogen receptor status. Lumican affected the expression levels of invadopodia markers, vinculin and talin and cortactin in the most invasive breast cancer cell lines. Vinculin role is crucial, as it interacts with integrins to the cytoskeleton at the focal adhesion complexes, and eventually controls the cytoskeletal mechanics, as well as lamellipodia formation. These proteins of the focal adhesion complexes were evaluated in the breast cancer cell lines in absence and presence of lumican. Vinculin expression was found to be increased in the highly invasive MDA-MB-231 cells, and moderately decreased in shERβMDA-MB-231 cells. Lumican downregulated

vinculin in both the highly metastatic MDA-MB-231 cells, as well as the shERβMDA-MB-231cells, is suggesting the anti-metastatic potential that lumican endows. Talin followed a similar profile, with profound effect of lumican in both MDA-MB-231 and shERβMDA-MB-231 cells. Using immunofluorescence, the ability of cortactin to create aggregates with actin leading to the initiation of invadopodia formation was observed in the highly metastatic MDA-MB-231 cells. Upon lumican treatment, cortactin expression was reduced, as well as the cytoplasmic and pericellular staining. Thus, lumican is able to inhibit the initiation of the formation of cellular protrusions by decreasing the expression of cortactin. As expected, in low invasive and low-metastatic MCF-7 cells, the staining of cortactin was very weak, in agreement with the low invasive and low metastatic potential of these cells. Presence of lumican rendered staining of cortactin even weaker, underlying the effect of lumican even in a low-invasive cell line (Karamanou *et al.*, submitted to FEBS J, [105]).

In addition, our group recently demonstrated that collagen-binding integrins, such as α1 and α2 in MDA-MB-231 cells, were downregulated in MCF-7 cells by the presence of lumican. It is quite interesting to note that the binding of integrins to focal adhesions transmits signals from the extracellular environment to the intracellular network, and inversely, mediated by integrins downstream signaling pathways, such as FAK, ERK1/2, MAPK 42/44, and AKT, which were found to be downregulated by lumican. Altogether our recent data suggest that lumican interacts through integrins, and downregulate FAK phosphorylation, which lead to the downregulation of phosphorylation of p130Cas and AKT. The decreased phosphorylation of p130Cas of the downstream signaling events results in the decrease of lamellipodia formation and MMP-14 activity, leading to an inhibition of cell migration.

Moreover, the expression of several matrix molecules, such as hyaluronan (HA), hyaluronan synthases (HASes), CD44 and invadopodia markers, integrins and signaling effectors was investigated by our group. All these findings, together with the observations by other researchers suggest that lumican effect is due to its ability to downregulate the phosphorylation of major cellular kinases of cell migration and proliferation/survival signaling, such as AKT, ERK, FAK [73, 81, 82, 106-108]. Conclusively, lumican interacts with integrins, downregulates FAK phosphorylation, which eventually results to the decreased phosphorylation of p130Cas and AKT. This downregulation of the above signaling cascade results to the decrease of the formation of cellular protrusions, such as lamellipodia, and MMP-14 activity, leading eventually to the inhibition of cell migration (Karamanou *et al.*, submitted to FEBS J, [105]). Figure 3 summarizes the effect of lumican on EMT, matrix molecules and RTKs.

Increased expression levels of MMP-14 are found to be localized at the surface of melanoma cells in primary tumors and especially in the invasive cellular protrusions [109, 110]. Snail is a major transcriptional factor in EMT, which is reported to be increased in

cancer tissues and closely correlated with cancer progression, including melanoma [111]. Snail also provokes EMT, increases migration, invasion, as well as MMP-14 activity. At the late-stage melanoma and during metastasis, the epithelial marker E-cadherin is significantly decreased and it has been reported that the inhibition of the Snail-triggered EMT leads to the inhibition of metastasis [112]. In parallel, lumican is reported to down regulate B16F1 melanoma cell lung metastasis [84]. In addition, the effect of lumican was investigated on the expression and activity of MMP-14 in Snail-transfected-B16F1 melanoma cells *in vitro* as well as its effects in melanoma metastasis *in vivo* in a mice model following screening of cancer implicated matrix effectors, invadopodia markers and intracellular signaling pathways. It was observed that lumican reduced the levels of SNAIL-induced cell proliferation and cell migration by blocking MMP-14, as well as melanoma primary tumor development. Therefore, a lumican-based therapy targeting SNAIL-induced MMP-14 activity could be beneficial for melanoma treatment, underlining the regulatory effect of lumican in EMT [112].

In ovarian cancer, lumican was also reported to be one of the genes that affects the Epithelial-to-Mesenchymal Transition (EMT) status. Recently, Jingjing Wu *et al.*, reported that HMGA2 is one of the few gene markers that can differentiate most type I (mesenchymal gene status) from type II (epithelial gene status) cancer cell lines [113]. Thuault *et al.*, also identified HMGA2 as a transcriptional regulator of SNAIL 1, a key EMT molecule. HMGA2 enhances tumor transformation in different cell types [114]. For instance, when it is overexpressed, it can be related with aggressive tumor growth, early metastasis and poor prognosis in several cases, such as pancreatic and breast cancer. When HMGA2 is repressed, the epithelial phenotype is restored with significant increase of E-cadherin. Similarly, in ovarian cancer cells, HMGA2 increases cell transformation. Several HMGA2-regulated genes were associated with EMT, one of which is lumican. Based on the literature regarding the anti-cancer effect of lumican and its role as tumor suppressor by inhibiting EMT, as well as identifying lumican as a target of HMGA2 in ovarian cancer, evidence is provided that HMGA2 promotes ovarian tumorigenesis through EMT regulation [113].

The role of lumican in EMT in response to injury is also reported [115]. It has been observed that lumican is immunolocalized in human postoperative capsular specimens. Detailed examination was followed using organ cultures of injured mouse lenses from both wild type and lumican knockout animals, to investigate the possibility that lumican modulates EMT of lens epithelial cells (LECs) in response to injury or to exposure to TGF-β2. The results obtained from wild type mice indicated that in uninjured lenses there was no lumican expression, whereas in lenses subjected to capsular injury, lumican protein was initially detected after 12h of culture and thereafter staining was gradually increased. The cells around the capsular break appeared elongated at day 5 and a fibroblast-like morphology may be assumed, indicating that EMT was in progress. Moreover, αSMA was detected in the fibroblast-like lens cells. In lumican knockout mice epithelial-shaped cells were present at

the same time point and αSMA was detected in lens cells at day 10, however at weaker intensity as compared with the wild type mice. By incubating the cultures with TGF-β2, strong lumican expression was detected at 24h, whereas in the absence of TGF-β2 only faint staining was observed. In addition, at day 10, wild type LECs cultured in the absence of TGF-β2 remained epithelial-like, and they become positive for lumican but remained negative for αSMA. In conclusion, the results taken together indicate that lumican was upregulated before EMT of the LECs and that loss of lumican attenuates injury-induced EMT of LECs [115].

Another case where lumican is reported to regulate EMT is the ventilation-induced EMT through extracellular signal-regulated kinase pathway [116]. In acute lung injury, mechanical ventilation is used in patients, since it damages pulmonary epithelial cells through production of inflammatory cytokines and excess deposition of lumican. The mechanisms underlying the interactions between mechanical ventilation and lung injury are not totally clarified. The main hypothesis is that lung damage and EMT upregulate lumican because of high tidal volume mechanical stretch and as a consequence, MIP-2 and TGF-β1 are modulated through the extracellular signal- regulated kinase (ERK 1/2 pathway). The experimental model used was C57BL/6 both wild-type and lumican knockout, exposed either to low or high tidal volume. It is worth noticing that high tidal volume signs activation of lumican and ERK 1/2 in wild-type mice, as well as decrease of E-cadherin staining. Conclusively, lumican promotes mechanical ventilation of high tidal volume, which induces lung injury and EMT through the activation of the ERK 1/2 pathway [116]. Lumican effect on invadopodia formation is presented in Table 1.

5) Clinical Benefits of the Anti-Cancer Effect of Lumican

The data of this review suggest that the treatment with lumican may serve for therapy of breast cancer. However, regarding the mechanisms of the anticancer effect of lumican, further studies are needed, and especially concerning the cell signaling that relates with invadopodia functions, in order to shed light in cancer metastasis treatment. Through mass-spectrometry-based proteomics, lumican was found to be unregulated in oral lichen planus (OLP-T) and oral squamous cell carcinoma (OSCC-T) groups in comparison with adjacent and control groups of patients. Therefore, lumican was identified as important pathogenesis biomarker of OLP that underlines its malignant potential [117].

One more article relates the lumican expression patterns and the clinical, pathological and oncological outcomes in patients with pancreatic ductal adenocarcinoma (PDAC), as well as the role of lumican in PDAC progression. Using microarray staining and COX regression analysis, it was reported that lumican was present in the stroma surrounding PDAC cells in mostly 50% of primary tumors and the direct xenografts. Patients with early stage of cancer and positive staining for stromal lumican were related with a profound decrease in metastatic recurrence after surgery and 3-times longer survival in comparison with patients

with negative staining for stromal lumican. Conclusively, there is a positive correlation between stromal lumican in primary PDAC tumors and prolonged survival after tumor resection [118].

Moreover, the prognostic value of lumican expression was also evaluated by clinicopathological data and tissue samples collected from stages II and III of colon cancer. Lumican expression in epithelial cells overall in the tumor was associated to a longer disease specific survival in stage II cancer patients, as well as a longer disease-free survival [119]. Lumican also serves as biomarker for metastatic and recurrent giant cell tumor of bone in lung cancer [120].

6) Conclusions and Perspectives

Invadopodia constitute an attractive target for metastasis promotion and inhibition. Data from our research group demonstrated that incubation of breast cancer cells with the anti-cancer effector lumican may inhibit or even reverse the several metastatic features that EMT endows. Since EMT is correlated with migration and invasion, as well as with the initiation of metastasis, a lumican-based anti-cancer therapy which will pharmacologically target and inhibit EMT might be interesting to be developed. Although lumican peptides might be susceptible to proteolytic degradation by the various proteases and could not be used easily for therapeutic purposes, protected derivatives and/or nanoformulations could be alternatives for their administration in solid tumors. Taking into consideration the growing field of nanotechnology and its raising applications in therapeutics, it may also be plausible to suggest that local overexpression of lumican in solid tumors using nanoformulations may be another useful approach or combined approaches could drive expression of lumican locally as a promising tool to consider for preventing invasion and metastasis at distant sites.

Conflict of Interest statement

The authors declare that there are no conflicts of interest**.**

Acknowledgements

We wish to thank Pr. F.-X. Maquart (University of Reims, Champagne-Ardenne, France) for the suggestions during the course of this work and Mr. Gianfranco Filippini DISTAL-Plant Pathology (University of Bologna, Italy). The work was co-financed by the Region Champagne-Ardenne, the Fond Européen de Développement Régional (FEDER), le contrat de projet Etat Région CPER 2012–2018, the Ligue contre le cancer, Conférence de Coordination Inter Régionale du Grand Est (CCIR-GE) 30036506-UMR7369 and the PHC Polonium. This publication is part of the joint-PhD (University of Patras/University of Reims Champagne Ardenne) of Konstantina Karamanou, under the financial support of Eiffel

Scholarship of Excellence (870731F), supplied from the French Ministry of Foreign Affairs, as well as General Secretariat for Research and Technology (GSRT) & the Hellenic Foundation for Research and Innovation (HFRI).

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Figures Legends

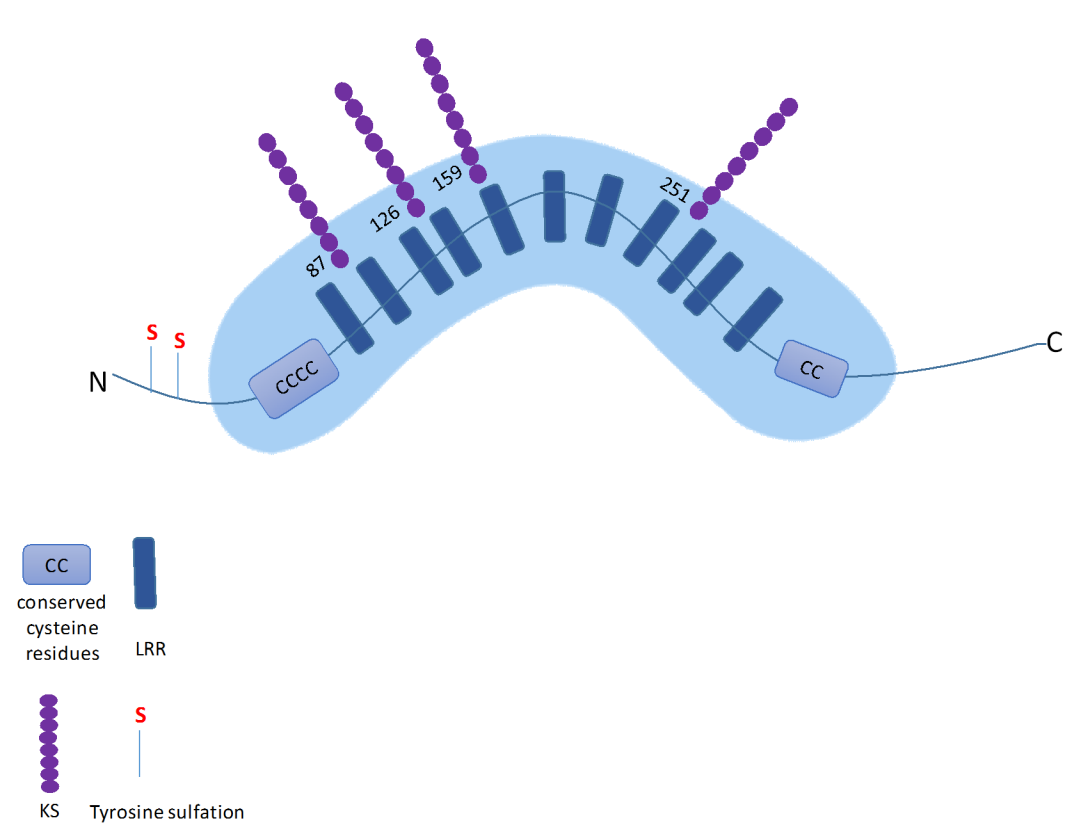
Figure 1 : Schematic representation of lumican structure. Lumican is a 338 amino acids protein. An 18 amino acid signal peptide is included, which permits the secretion of lumican into ECM, as well as three major domains: a) a negatively-charged N-terminal domain containing four cysteines with disulfide bonds and potential sites for tyrosine sulfation, b) a central part which is highly conserved and is described to contain 11 leucine rich repeats, c) a C-terminal domain of 66 amino acids which contains two conserved cysteines and two LRRs. The LRRs motifs are shown as blue boxes, and numbered from N-terminal to C-terminal. There are four potential sites for substitution by N-linked KS or oligosaccharides, situated at position 87, 126, 159, 251 of the core protein of human lumican.

Figure 2 : Observation of MDA-MB-231 cells by SEM, set on Millipore® filter coated with type I collagen. First panel (images 1,2): MDA-MB-231 cells cultured for 24h, appeared with mesenchymal phenotype, spindle-like shape and a plethora of cellular protrusions, like lamellipodia or filopodia. The arrow in image 1 depicts the spindle-like shape. In image 2, 100 nM lumican seems to endow MDA-MB-231 cells with a more rounded shape and diminished number of cellular protrusions. Second panel (images 3,4): MDA-MB-231 cultured for 48h. The mesenchymal morphology is still evident, although cells seem to be more flattened. In image 4, 100nM lumican rendered cells with a more globular shape and the cell surface seemed smooth.

Figure 3 : Schematic representation of lumican effect in EMT, focal adhesion proteins, cellular functions and signaling pathways. Highly-invasive, mesenchymal MDA-MB-231 cells are transformed into epithelial-like upon 100nM lumican treatment after 48h of cell culture. Lumican diminishes the number of cellular protrusions, since it decreases the expression of cortactin; major protein of invadopodia formation, and vinculin; protein of focal adhesions. Lumican also affects the activity and expression of MMP-14, and as a consequence cellular migration, invasion, and several RTKs, such as FAK signaling pathway. The structure of invadopodium is presented in the insert. MMP-14 is depicted on the edge of invadopodium, facilitating migration and invasion. Vinculin and talin are essential for the integrins connection to cytoskeleton.

Table 1. Lumican effect on EMT and invadopodia formation according to cancer.

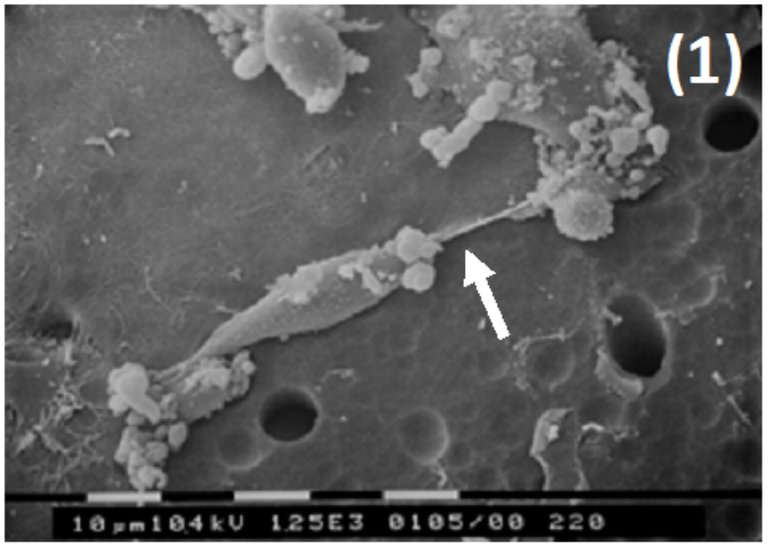
Pathological condition	Effect on EMT	Effect on invadopodia	References
Bone-derived prostate cancer	♦ Re-organization of keratin 8 and 18 into pericellular localization ♦ Destabilization of focal complexes ♦ Decrease of ZO-1 expression	♦ Lamellipodia and invadopodia are diminished	[98]
Highly-invasive breast cancer	♦ Altered expression of invadopodia markers (vinculin, talin, cortactin) ♦ Heterogeneity in cell populations (co-existence of spindle-like and flattened cells) ♦ Tendency to regain an epithelial phenotype	♦ Decrease in the number of lamellipodia and filopodia	Karamanou *et al.*, submitted to FEBS J, [105]
Low-invasive breast cancer	♦ Morphological alterations ♦ Globularly-shaped cells ♦ Altered gene expression of EMT markers (increase of E-cadherin, decrease of vimentin)	♦ Diminished number of invadopodia and absence of microvilli, microvesicles and invadosomes ♦ Release of exosomes	Karamanou *et al.*, submitted to FEBS J, [105]
Melanoma	♦ Cell migration is affected ♦ Expression of invadopodia markers is altered ♦ Blocking of MMP-14 activity	♦ Invasive cellular protrusions are affected	[109-112]
Ovarian Cancer	♦ HMGA2, key EMT molecule is affected ♦ Promotion of tumorigenesis through EMT regulation	Not reported	[113]
Lung injury	♦ Promotion of mechanical ventilation of high tidal volume ♦ Induction of EMT through ERK 1/2 pathway ♦ MIP-2 and TGF-beta expression are modulated	Not reported	[116]

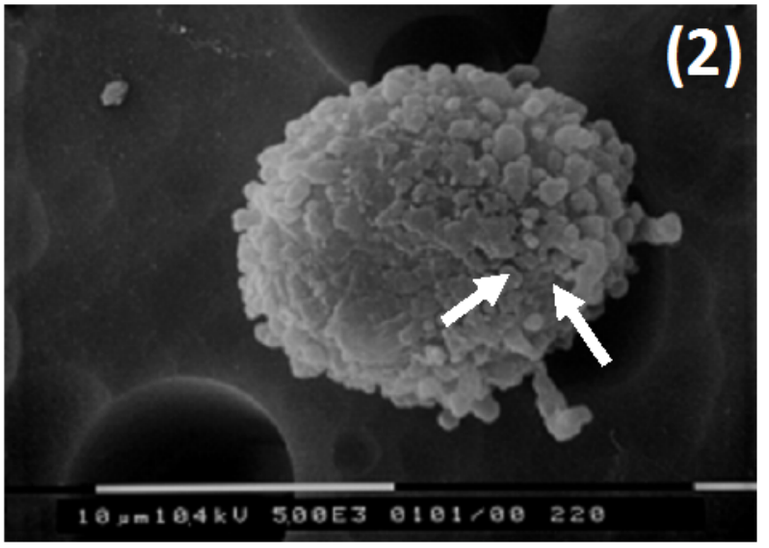

87
126
159
251
S
S
N
CCCC
CC
C
CC
conserved cysteine residues
LRR
KS
S
Tyrosine sulfation

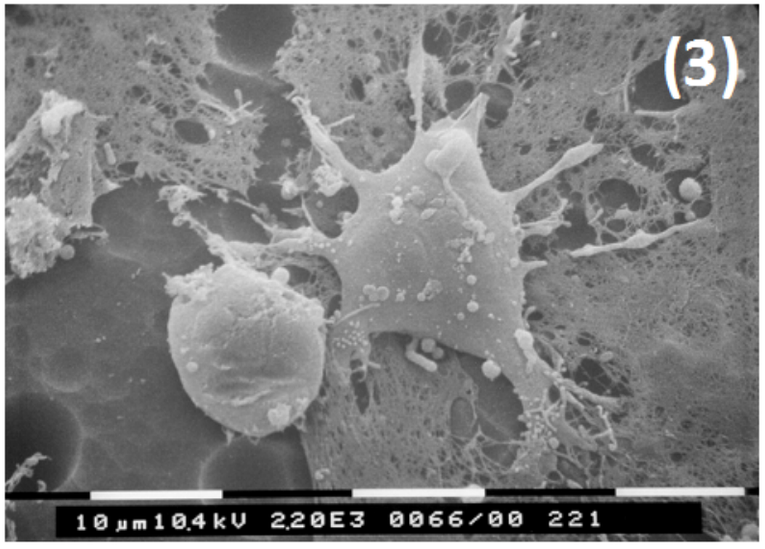
24h

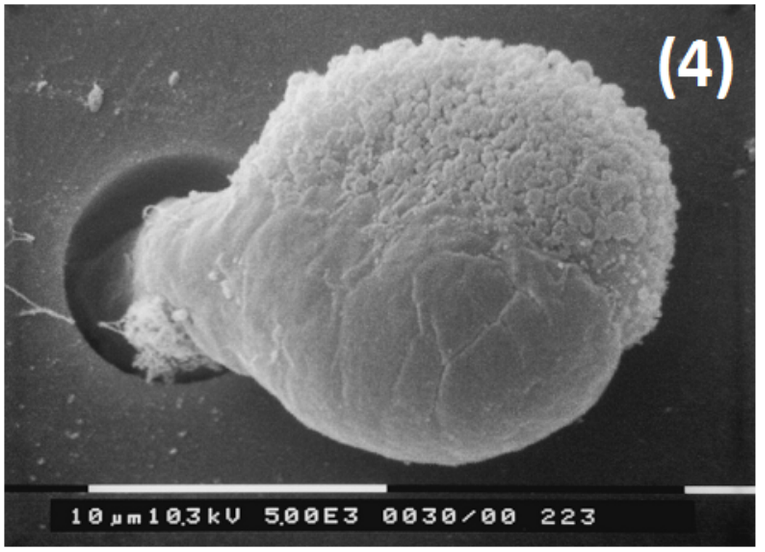
MDA-MB-231

MDA-MB-231
+Lumican





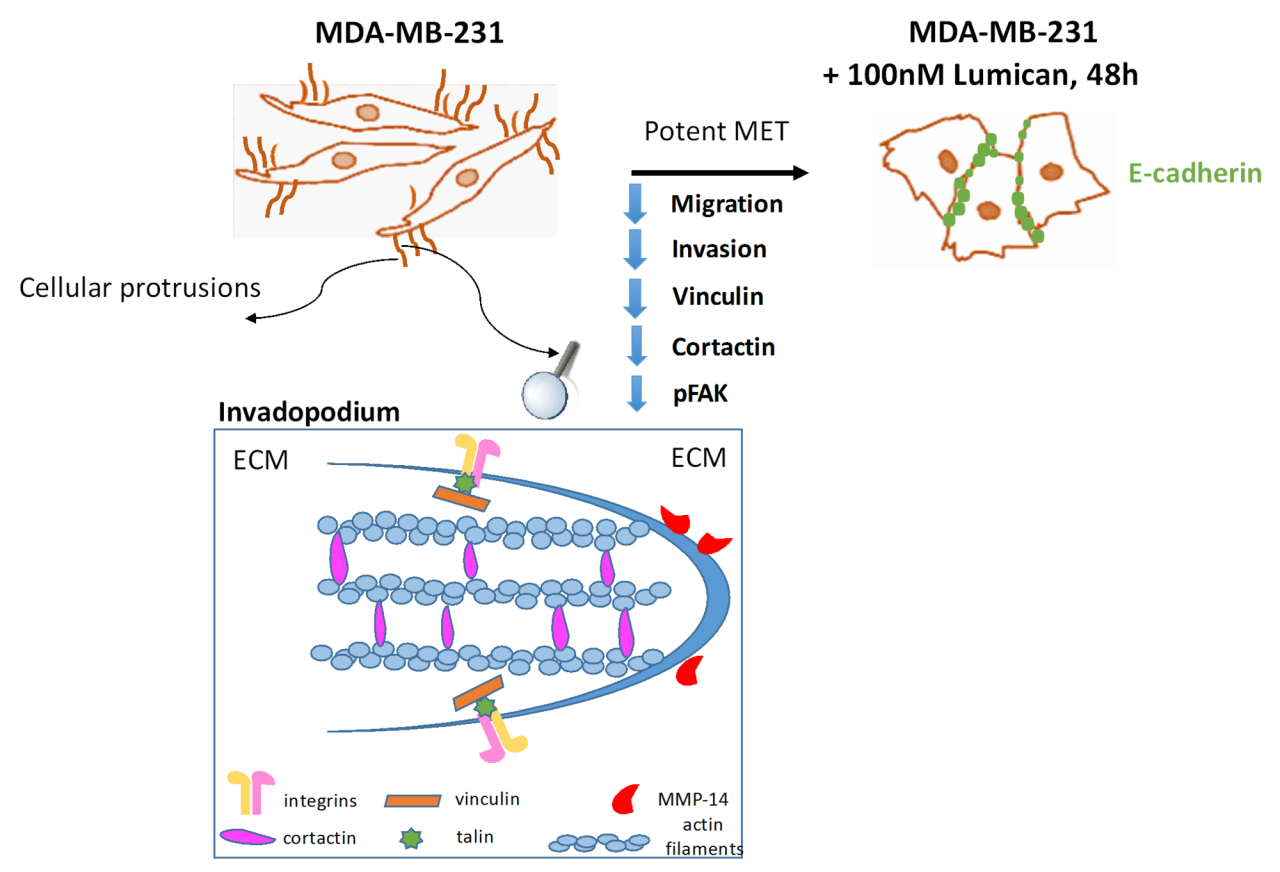




MDA-MB-231

MDA-MB-231
+Lumican

48h


MDA-MB-231
MDA-MB-231
+ 100nM Lumican, 48h
Potent MET
Migration
Invasion
Vinculin
Cortactin
pFAK
E-cadherin
Cellular protrusions
Invadopodium
ECM
ECM
integrins
vinculin
MMP-14
cortactin
talin
actin
filaments