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► To cite this version:

Konstantina Karamanou, Marco Franchi, Demitrios Vynios, Stéphane Brézillon. Epithelial-to-mesenchymal transition and invadopodia markers in breast cancer: Lumican a key regulator. *Seminars in Cancer Biology*, 2020, 62, pp.125-133. 10.1016/j.semcancer.2019.08.003 . hal-03052108

HAL Id: hal-03052108

<https://hal.univ-reims.fr/hal-03052108v1>

Submitted on 20 May 2022

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1 **Epithelial-to-Mesenchymal Transition and invadopodia markers in**
2 **breast cancer: Lumican a key regulator**

3

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19

20 **Abstract**

21

22 A great hallmark of breast cancer is the absence or presence of estrogen receptors ER α and
23 ER β , with a dominant role in cell proliferation, differentiation and cancer progression. Both
24 receptors are related with Epithelial-to-Mesenchymal Transition (EMT) since there is a
25 relation between ERs and extracellular matrix (ECM) macromolecules expression, and
26 therefore, cell-cell and cell-ECM interactions. The endocrine resistance of ER α endows
27 epithelial cells with increased aggressiveness and induces cell proliferation, resulting into a
28 mesenchymal phenotype and an EMT status. ER α signaling may affect the transcriptional
29 factors which govern EMT. Knockdown or silencing of ER α and ER β in MCF-7 and MDA-MB-
30 231 breast cancer cells respectively, provoked pivotal changes in phenotype, cellular
31 functions, mRNA and protein levels of EMT markers, and consequently the EMT status.
32 Mesenchymal cells owe their migratory and invasive properties to invadopodia, while in
33 epithelial cells, lamellipodia and filopodia are mostly observed. Invadopodia, are actin-rich
34 protrusions of plasma membrane, promoting proteolytic degradation of ECM and tumor
35 invasion. Cortactin and MMP-14 govern the formation and principal functions of
36 invadopodia. *In vitro* experiments proved that lumican inhibits cortactin and MMP-14
37 expression, alters the formation of lamellipodia and transforms mesenchymal cells into
38 epithelial-like. Conclusively, lumican may inhibit or even reverse the several metastatic
39 features that EMT endows in breast cancer cells. Therefore, a lumican-based anti-cancer
40 therapy which will pharmacologically target and inhibit EMT might be interesting to be
41 developed.

42

43 **Keywords**

44

45 EMT; invadopodia; breast cancer; lumican

46

47

48

49 **1) Cell migration and invadopodia**

50

51 Cell migration is a mechanical physiological process occurring during embryogenic
52 morphogenesis, bone homeostasis, tissue repair and regeneration. Cell invasion consisting
53 in the breaching of tissue barriers like endothelial basement membrane is a basic function of
54 immune cells to respond and to prevent infections. Invasion takes place during disease
55 progression such as cancer invasion of extracellular matrix (ECM) and final metastasis [1].

56 There are distinct types of cell migration: cells can migrate as individual cells or collectively
57 in group of cells moving together, just retained by intercellular interactions. Cells can move
58 in two different mechanical ways referred to “amoeboid” or “mesenchymal” movements [2,
59 3]. In general, amoeboid movement is protease-independent, but requires pores or
60 channels (more than 3-5 μm in diameter) for cells to squeeze through, whereas
61 mesenchymal movement is protease-dependent and is necessary to traverse nanoporous
62 matrices such as basement membranes [4, 5].

63

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

89

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

99

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

109

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

114

115 Podosomes are actin structures connecting the cytoskeleton with the plasma membrane
116 and appear like small plasma membrane protrusions distributed at the cell surface,
117 measuring only 0.4 μm in length and 1 μm in diameter and undergoing constant
118 disassembly and reformation [28, 29]. They are associated with normal cells, such as
119 macrophages, osteoclasts, dendritic cells, epithelial cells, smooth muscle cells and
120 fibroblasts and consist in a branched actin core containing proteins involved in actin
121 polymerization and a surrounding ring rich in $\beta 2$ and $\beta 3$ integrin receptors and adhesion
122 proteins such as talin, vinculin and paxillin [3, 4, 30, 31]. Podosomes act like probing or
123 palpating organelles well distributed on cell membrane of the myelomonocytic lineage, such
124 as macrophages [32], immature dendritic cells [33] and osteoclasts [34]. They are mainly
125 related to cell migration and diapedesis of blood leukocytes even though they may assume
126 an invasive function in facilitating the tumor cell invasion process by a proteolytic activity
127 [22, 29, 35, 36].

128

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

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

139

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

149

150 **2) Relation of invadopodia with cancer**

151

152 Invadopodia are described in invading cells, such as metastatic tumor cells [39, 44]. In fact,
153 they are always closely located to the ECM and represent enzymatically matrix-degrading
154 structures burrowing across tissue barriers through the release of matrix metalloproteases
155 (MMPs) which mainly include MMP-14, MMP-2 and MMP-9 [12, 16, 39, 40]. These MMPs,
156 and in particular MMP-14, are secreted to the site of invadopodia adhesion through vesicles
157 [45]. Even though the main function of invadopodia in tumors is to promote the proteolytic
158 matrix degradation to favor cancer cell invasion, other functions have been suggested: the
159 proteolytic action of these cytoplasmic protrusions to create space in the ECM could favor
160 tumors growth or alternatively the pericellular proteolytic activity at invadopodia surface
161 could activate various growth factors to drive tumor growth [43] and favor angiogenesis [46,
162 47].

163

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

172 One major protein which is required for the assembly of invadopodia by regulating the F-
173 actin-enriched invadopodial cytoskeleton is cortactin, a substrate of Src family tyrosine
174 kinases. By promoting invadopodia formation, function and disassembly, it is related to
175 cancer cell migration, invasion, intravasation, extravasation and metastasis [49-53]. An
176 overexpression of cortactin is frequently reported in several types of invasive cancers so
177 that it is widely used as a marker of invadosomes [48]. Similarly, inhibition of cortactin leads
178 to the inhibition of invadopodia formation and reduces metastasis to distant organs [54,
179 55].

180

181 Different morphologic conformations of invadosomes such as aggregates, individual dots,
182 rosettes or linear shaped structures along type I collagen fibrils can be observed in the same
183 cell, thus supporting the concept of invadosome plasticity [22]. The relationship between
184 podosomes and invadopodia is not clearly defined in the literature: it was also suggested
185 that podosomes and invadopodia may share a common primordial precursor able to adapt
186 to its microenvironment and modulate its morphology in relation to the different substrates
187 [39, 44, 56, 57]. Moreover, a close distinction in function between pseudopodia and
188 invadosomes seems to be discussed: some authors suggested that a high degree of
189 molecular integration and cross talk between pseudopodia and invadopodia allowing
190 efficient invasion coupled migration in both 2 and 3D may be also possible [27, 58].
191 Formation of both invasive protrusions (invadopodia) and locomotory protrusions
192 (pseudopodia) is regulated by stromal cells in the tumor microenvironment like fibroblasts
193 in squamous cell carcinoma [59] and macrophages in breast carcinoma [60, 61]. Moreover,
194 ECM rigidity seems to influence the formation of invadopodia so that alterations in matrix
195 stiffness may be related to cancer disease and progression [61]. It is worth noticing that
196 dense fibrillar collagen so as transient mechanical strains promote the maturation of
197 invadopodia and enhances cancer cell invasion *in vitro* [40, 42]. In particular, it was
198 demonstrated that fibrillar collagen I is a physiological inducer of a novel class of
199 invadosomes called "linear invadosomes" which might act as collagen I fibril sensors and are
200 able to remodel the ECM [62]. In addition, hypoxia of peritumoral stroma can promote
201 mesenchymal invasion in breast cancer cells through the upregulation of structural
202 components of the actin cytoskeleton machinery involved in invadopodium formation [63].

203

204 **3) Lumican, a Class II Small Leucine Rich Proteoglycan, as a regulator of Epithelial-to-** 205 **Mesenchymal Transition**

206

207 ECM is a three-dimensional network of macromolecules which provides structural and
208 biochemical support to the surrounding cells. ECM also regulates intracellular
209 communication and affects cell behavior. Through the fine-tuned interactions between cell
210 surface receptors and ECM components, gene expression and diverse functional properties
211 are affected. ECM remodeling can occur in both physiological and pathological conditions
212 [64].

213 Proteoglycans (PGs) are key components of the ECM. PGs are constituted by a core protein
214 to which linear and highly anionic chains of glycosaminoglycans (GAGs) are covalently
215 attached. PGs are so called as multifunctional key effectors, since they are involved in a
216 plethora of pathophysiological processes, such as cancer [65-67]. PGs expression is
217 remarkably altered during tumor development and growth and their remodeling on the
218 tumor ECM and cell membranes influences major cancer cell properties, such as cell
219 proliferation, migration, invasion, angiogenesis and adhesion [68]. The most abundantly
220 expressed PGs in ECM are the Small Leucine-Rich PGs (SLRPs). Their organization is
221 pericellular and their core protein substituted by negatively charged GAG chains enable the
222 interactions of SLRPs with matrix effectors, such as cytokines, growth factors and cell
223 surface receptors. These interactions lead to the regulation of crucial cell functional
224 features, i.e.: migration, autophagy, angiogenesis and metastatic potential of cancer cells
225 [69-71]. SLRPs comprise of 18 members classified into five classes according to the
226 conservation and homology at the protein and genomic levels. Lumican is a Class II SLRP,
227 characterized by high molecular heterogeneity according to the tissue due to its
228 glycosylation. Human lumican core protein is a protein of 338 amino acids, including an 18
229 amino acid signal peptide and three major domains, a negatively charged N-terminal
230 domain containing cysteine and sulfated tyrosine residues, a central part containing 9
231 Leucine Rich Repeats (LRR), a terminal domain of 66 amino acids containing 2 conserved
232 cysteine and 2 LRRs. The structure of lumican is illustrated in Figure 1.

233
234 Lumican expression is abundant in the ECM of many tissues, such as skin, kidney, breast,
235 colon, pancreas, cartilage, etc. [72]. In certain tissues, lumican is linked positively with
236 tumor aggressiveness, while in some other tissues, negatively [73]. In breast carcinoma, as
237 well as in melanoma, lumican expression is increased in the stroma [74-78]. Increased
238 expression of lumican in the stroma of breast carcinomas is not a prognostic factor for
239 breast cancer. However, enhanced lumican expression is related with increased levels of
240 metastasis, decreased levels of estrogen receptors (ERs) expression and the young age of
241 the patients. On the other hand, the decreased lumican expression is reported to be related
242 with poor clinical outcome and survival of the patient [76, 78].

243
244 Several published studies refer to the anti-cancer action of lumican, although the anti-
245 tumorigenic mechanism behind it, is still not totally clarified. Data from our research group
246 have suggested an anti-cancer effect of lumican in melanoma. *In vitro*, the core protein of
247 lumican (37 kDa) was reported to increase melanoma cell adhesion [79], and its glycosylated
248 form (57 kDa) inhibited cell migration and invasion, with simultaneous changes in the actin
249 network and focal adhesion complexes [80-83]. At *in vivo* level, when syngeneic mice were
250 injected with B16F1 melanoma cells stably transfected with lumican expressing plasmid
251 (Lum-B16F1), the size and the number of the lung metastatic nodules were significantly
252 decreased in comparison with the Mock-B16F1 cells [84]. Angiogenesis was also altered, as
253 the number of the blood vessels in the metastatic nodules was decreased [85].

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

270

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

279

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

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

297

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

311

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

323

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

332

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

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

346

347 **4) Lumican as an effector on invadopodia formation**

348

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

359

360 Keratin 8 and 18 are expressed separately in normal keratinocytes, however they are found
361 to be co-expressed in planocellular cancer cells, in the form of 8/18 filaments. This co-
362 expression of keratin 8/18 filaments enables the invasion of normal keratinocytes in the
363 basement membrane [99]. It is already reported that keratin 8 re-organization provokes
364 increased levels of cell motility, high levels of keratin 8 and 18 are signals of metastatic
365 progression and poor clinical outcome in squamous cell carcinomas (SCCs) [100-102]. On the
366 other hand, decreased expression of keratin 8 and keratin 18 have been correlated with low
367 $\alpha 6 \beta 4$ integrin expression, cancer cell migration and invasion [103]. When Coulson-Thomas
368 *et al.*, seeded prostate cancer cells upon lumican coating, both keratin 8 and 18 expression
369 was decreased, as well as their organization from cellular protrusions was modified into
370 perinuclear localization [98]. In addition, upon lumican treatment, de-stabilization of focal
371 adhesion complexes was observed to lead to inhibition of cell migration. More specifically,
372 Zona occludens protein 1 (ZO-1), a protein which plays pivotal role in cell migration, since it
373 is localized in lamellipodia and in intracellular tight junctions, presented a decreased
374 expression and was concentrated in the cell membrane and not in the cellular protrusions
375 [104].

376

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

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

392
393 Morphological changes that cells undergo after invasion were evaluated after seeding onto
394 different matrix-coated surfaces. Cells were cultured for both 24 and 48 hours and seeded
395 on the upper surface of a Millipore filter coated with collagen type I and were observed by
396 Scanning Electron Microscopy (SEM) (Figure 2). Regarding MDA-MB-231 breast cancer cells,
397 SEM analysis revealed their elongated, spindle-like shape, as well as their numerous
398 cytoplasmic protrusions. The cellular surface of MDA-MB-231 was irregular and non-
399 smooth. Upon 100nM lumican treatment in serum-free conditions for 48h, MDA-MB-231
400 breast cancer cells seemed more flattened with a smoother surface and a decreased
401 number of invadopodia, as seen in Figure 1. Similarly, 48h lumican treatment evoked
402 morphological changes in MCF-7 breast cancer cells during invasion in different collagen
403 substrates. SEM analysis revealed the flattened and globular morphology of MCF-7 cells, as
404 well as a diminished number of invadopodia and even few microvilli. After 24h of treatment
405 with 100nM lumican, SEM revealed morphological alterations in MCF-7, as they appeared as
406 more grouped more globularly-shaped. The cytoplasmic membrane was observed as
407 smooth with absence of microvilli, microvesicles and invadosomes. Exosomes were also
408 observed (Karamanou *et al.*, submitted to FEBS J, [105]).

409
410 As already referred, invadopodia are membrane protrusions of invasive cancer cells,
411 involved in the focal pericellular degradation of ECM. It is very critical to evaluate the three
412 breast cancer cell lines of different estrogen receptor status. Lumican affected the
413 expression levels of invadopodia markers, vinculin and talin and cortactin in the most
414 invasive breast cancer cell lines. Vinculin role is crucial, as it interacts with integrins to the
415 cytoskeleton at the focal adhesion complexes, and eventually controls the cytoskeletal
416 mechanics, as well as lamellipodia formation. These proteins of the focal adhesion
417 complexes were evaluated in the breast cancer cell lines in absence and presence of
418 lumican. Vinculin expression was found to be increased in the highly invasive MDA-MB-231
419 cells, and moderately decreased in shER β MDA-MB-231 cells. Lumican downregulated

420 vinculin in both the highly metastatic MDA-MB-231 cells, as well as the shER β MDA-MB-
421 231 cells, is suggesting the anti-metastatic potential that lumican endows. Talin followed a
422 similar profile, with profound effect of lumican in both MDA-MB-231 and shER β MDA-MB-
423 231 cells. Using immunofluorescence, the ability of cortactin to create aggregates with actin
424 leading to the initiation of invadopodia formation was observed in the highly metastatic
425 MDA-MB-231 cells. Upon lumican treatment, cortactin expression was reduced, as well as
426 the cytoplasmic and pericellular staining. Thus, lumican is able to inhibit the initiation of the
427 formation of cellular protrusions by decreasing the expression of cortactin. As expected, in
428 low invasive and low-metastatic MCF-7 cells, the staining of cortactin was very weak, in
429 agreement with the low invasive and low metastatic potential of these cells. Presence of
430 lumican rendered staining of cortactin even weaker, underlying the effect of lumican even
431 in a low-invasive cell line (Karamanou *et al.*, submitted to FEBS J, [105]).

432

433 In addition, our group recently demonstrated that collagen-binding integrins, such as α 1
434 and α 2 in MDA-MB-231 cells, were downregulated in MCF-7 cells by the presence of
435 lumican. It is quite interesting to note that the binding of integrins to focal adhesions
436 transmits signals from the extracellular environment to the intracellular network, and
437 inversely, mediated by integrins downstream signaling pathways, such as FAK, ERK1/2,
438 MAPK 42/44, and AKT, which were found to be downregulated by lumican. Altogether our
439 recent data suggest that lumican interacts through integrins, and downregulate FAK
440 phosphorylation, which lead to the downregulation of phosphorylation of p130Cas and AKT.
441 The decreased phosphorylation of p130Cas of the downstream signaling events results in
442 the decrease of lamellipodia formation and MMP-14 activity, leading to an inhibition of cell
443 migration.

444

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

457

458 Increased expression levels of MMP-14 are found to be localized at the surface of
459 melanoma cells in primary tumors and especially in the invasive cellular protrusions [109,
460 110]. Snail is a major transcriptional factor in EMT, which is reported to be increased in

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

475
476 In ovarian cancer, lumican was also reported to be one of the genes that affects the
477 Epithelial-to-Mesenchymal Transition (EMT) status. Recently, Jingjing Wu *et al.*, reported
478 that HMGA2 is one of the few gene markers that can differentiate most type I
479 (mesenchymal gene status) from type II (epithelial gene status) cancer cell lines [113].
480 Thuault *et al.*, also identified HMGA2 as a transcriptional regulator of SNAIL 1, a key EMT
481 molecule. HMGA2 enhances tumor transformation in different cell types [114]. For instance,
482 when it is overexpressed, it can be related with aggressive tumor growth, early metastasis
483 and poor prognosis in several cases, such as pancreatic and breast cancer. When HMGA2 is
484 repressed, the epithelial phenotype is restored with significant increase of E-cadherin.
485 Similarly, in ovarian cancer cells, HMGA2 increases cell transformation. Several HMGA2-
486 regulated genes were associated with EMT, one of which is lumican. Based on the literature
487 regarding the anti-cancer effect of lumican and its role as tumor suppressor by inhibiting
488 EMT, as well as identifying lumican as a target of HMGA2 in ovarian cancer, evidence is
489 provided that HMGA2 promotes ovarian tumorigenesis through EMT regulation [113].

490
491 The role of lumican in EMT in response to injury is also reported [115]. It has been observed
492 that lumican is immunolocalized in human postoperative capsular specimens. Detailed
493 examination was followed using organ cultures of injured mouse lenses from both wild type
494 and lumican knockout animals, to investigate the possibility that lumican modulates EMT of
495 lens epithelial cells (LECs) in response to injury or to exposure to TGF- β 2. The results
496 obtained from wild type mice indicated that in uninjured lenses there was no lumican
497 expression, whereas in lenses subjected to capsular injury, lumican protein was initially
498 detected after 12h of culture and thereafter staining was gradually increased. The cells
499 around the capsular break appeared elongated at day 5 and a fibroblast-like morphology
500 may be assumed, indicating that EMT was in progress. Moreover, α SMA was detected in the
501 fibroblast-like lens cells. In lumican knockout mice epithelial-shaped cells were present at

502 the same time point and α SMA was detected in lens cells at day 10, however at weaker
503 intensity as compared with the wild type mice. By incubating the cultures with TGF- β 2,
504 strong lumican expression was detected at 24h, whereas in the absence of TGF- β 2 only faint
505 staining was observed. In addition, at day 10, wild type LECs cultured in the absence of TGF-
506 β 2 remained epithelial-like, and they become positive for lumican but remained negative for
507 α SMA. In conclusion, the results taken together indicate that lumican was upregulated
508 before EMT of the LECs and that loss of lumican attenuates injury-induced EMT of LECs
509 [115].

510

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

525

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

527

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

536

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

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

547

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

554

555 **6) Conclusions and Perspectives**

556

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

571

572 **Conflict of Interest statement**

573

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

575

576 **Acknowledgements**

577

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

586 Scholarship of Excellence (870731F), supplied from the French Ministry of Foreign Affairs, as
587 well as General Secretariat for Research and Technology (GSRT) & the Hellenic Foundation
588 for Research and Innovation (HFRI).
589

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- 864
- 865

866 **Figures Legends**

867

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

877

878 **Figure 2 : Observation of MDA-MB-231 cells by SEM, set on Millipore® filter coated with**
879 **type I collagen.** First panel (images 1,2): MDA-MB-231 cells cultured for 24h, appeared with
880 mesenchymal phenotype, spindle-like shape and a plethora of cellular protrusions, like
881 lamellipodia or filopodia. The arrow in image 1 depicts the spindle-like shape. In image 2,
882 100 nM lumican seems to endow MDA-MB-231 cells with a more rounded shape and
883 diminished number of cellular protrusions. Second panel (images 3,4): MDA-MB-231
884 cultured for 48h. The mesenchymal morphology is still evident, although cells seem to be
885 more flattened. In image 4, 100nM lumican rendered cells with a more globular shape and
886 the cell surface seemed smooth.

887

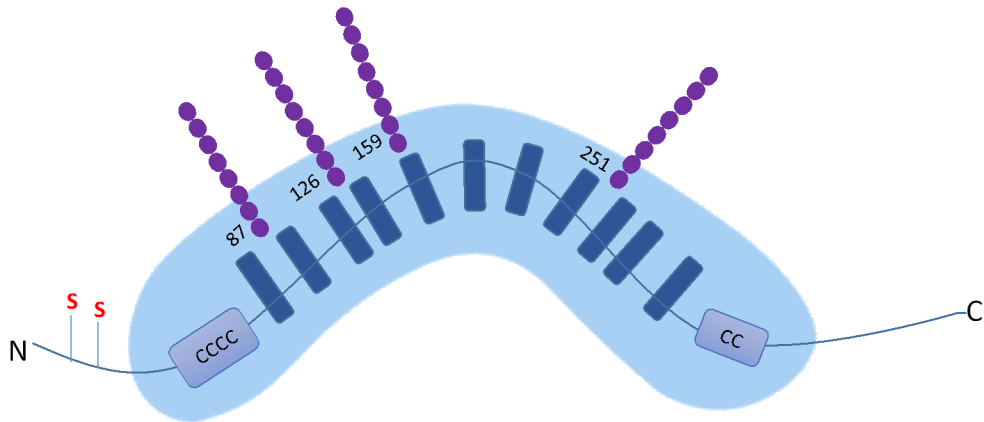
888 **Figure 3 : Schematic representation of lumican effect in EMT, focal adhesion proteins,**
889 **cellular functions and signaling pathways.** Highly-invasive, mesenchymal MDA-MB-231 cells
890 are transformed into epithelial-like upon 100nM lumican treatment after 48h of cell culture.
891 Lumican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

898

899 **Table 1.** Lumican effect on EMT and invadopodia formation according to cancer.
 900

| Pathological condition | Effect on EMT | Effect on invadopodia | References |
|-------------------------------|--|--|--|
| Bone-derived prostate cancer | <ul style="list-style-type: none"> ◆ Re-organization of keratin 8 and 18 into pericellular localization ◆ Destabilization of focal complexes ◆ Decrease of ZO-1 expression | <ul style="list-style-type: none"> ◆ Lamellipodia and invadopodia are diminished | [98] |
| Highly-invasive breast cancer | <ul style="list-style-type: none"> ◆ 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 | <ul style="list-style-type: none"> ◆ Decrease in the number of lamellipodia and filopodia | Karamanou <i>et al.</i> , submitted to FEBS J, [105] |
| Low-invasive breast cancer | <ul style="list-style-type: none"> ◆ Morphological alterations ◆ Globularly-shaped cells ◆ Altered gene expression of EMT markers (increase of E-cadherin, decrease of vimentin) | <ul style="list-style-type: none"> ◆ Diminished number of invadopodia and absence of microvilli, microvesicles and invadosomes ◆ Release of exosomes | Karamanou <i>et al.</i> , submitted to FEBS J, [105] |
| Melanoma | <ul style="list-style-type: none"> ◆ Cell migration is affected ◆ Expression of invadopodia markers is altered ◆ Blocking of MMP-14 activity | <ul style="list-style-type: none"> ◆ Invasive cellular protrusions are affected | [109-112] |
| Ovarian Cancer | <ul style="list-style-type: none"> ◆ HMGA2, key EMT molecule is affected ◆ Promotion of tumorigenesis through EMT regulation | Not reported | [113] |
| Lung injury | <ul style="list-style-type: none"> ◆ 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] |

901



CC
conserved
cysteine
residues

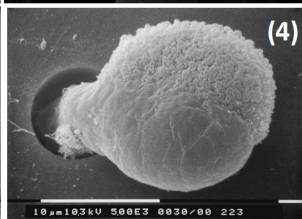
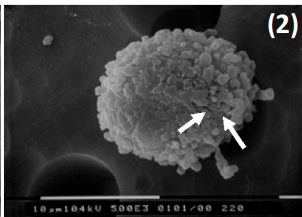
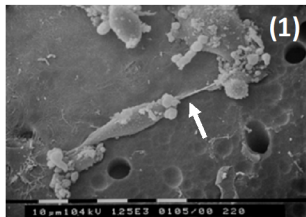
LRR

KS
Tyrosine sulfation

24h

MDA-MB-231

MDA-MB-231
+Lumican



MDA-MB-231

MDA-MB-231
+Lumican

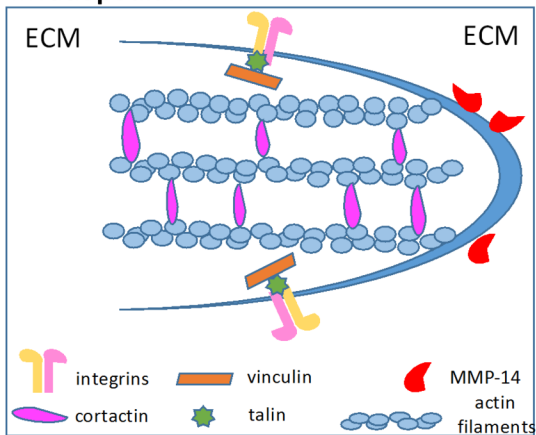
48h

MDA-MB-231



Cellular protrusions

Invadopodium



Potent MET



Migration

Invasion

Vinculin

Cortactin

pFAK



MDA-MB-231 + 100nM Lumican, 48h



E-cadherin