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Extracellular matrix endocytosis in controlling matrix turnover and beyond: emerging roles in cancer

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Abbreviations: ECM=extracellular matrix; MMP=matrix metalloproteases; BM=basement membrane; DG=dystroglycan; MR=mannose receptor; NMMIIA= non-muscular myosin II A; EE=early endosome; Lys=lysosome; FN=fibronectin; LN=laminin; PSC=pancreatic stellate cells

Abstract

The extracellular matrix (ECM) is a network of secreted proteins that, beyond providing support for tissues and organs, is involved in the regulation of a variety of cell functions, including cell proliferation, polarity, migration and oncogenic transformation. ECM homeostasis is maintained through a tightly controlled balance between synthesis, deposition and degradation. While the role of metalloproteases (MMPs) in ECM degradation is widely recognised, the contribution of ECM internalisation and intracellular degradation to ECM maintenance has been mostly overlooked. In this review, I will summarise what is known about the molecular mechanisms mediating ECM endocytosis and how this process impacts on diseases, such as fibrosis and cancer.

Introduction

The extracellular matrix (ECM) is a complex 3 dimensional (3D) network of secreted proteins. The "matrisome project" identified ~300 proteins as core matrisome components [1]. These include collagens, proteoglycans (including aggrecan, versican and perlecan) and glycoproteins (such as laminins, fibronectin and nidogen). The ECM comes in 2 "flavours": the interstitial connective tissue, which offers structural scaffolding for tissues, and the basement membrane (BM), which mediates the separation between the epithelium and the stroma (**figure 1**).

The ECM is constantly remodelled, as a result of a fine balance between synthesis, deposition and degradation. The functions of the ECM go beyond providing tissue support. Indeed, the ECM is implicated in the control of tissue homeostasis and epithelial morphogenesis, while deregulation of ECM remodelling has been linked to several pathological conditions [2]. On one hand, excessive ECM degradation leads to tissue destruction in cardiomyopathies and osteoarthritis, indicating the need for an intact ECM network to support tissue architecture and homeostasis. On the other hand, ECM accumulation is associated with fibrosis and cancer [2]. Cells interact with the ECM through plasma membrane receptors, including integrins, dystroglycan (DG), discoid domain receptors (DDRs) and mannose receptors (MRs). These are involved in a variety of cell functions, such as proliferation, migration and polarity [3].

Two main ECM degradation pathways have been described: extracellular or pericellular degradation, mediated by matrix metalloproteases (MMPs), and lysosomal degradation after receptor-mediated internalisation. These are not independent processes, and are more likely to occur in a coordinated

fashion, as detailed below. This short review focuses on the molecular mechanisms responsible for ECM internalisation and how this process impinges on pathological conditions, such as fibrosis and cancer.

Mechanisms controlling ECM internalisation

Collagens are the most abundant components of the ECM, accounting for up to 30% of the total proteins in human, [3]. Collagen internalisation and degradation is an important step for connective tissue homeostasis and is required for physiological collagen turnover. So far, two mechanisms have been identified to control collagen internalisation: integrin-mediated phagocytic uptake and Endo180-dependent internalisation.

α2β1 integrin mediates collagen phagocytosis

Integrins are heterodimers composed of an α and a β subunit and provide a link between the ECM and the intracellular cytoskeleton [4]. The McCulloch group has identified α2β1 integrin as the receptor that mediates collagen I binding in fibroblasts and delineated the molecular pathway responsible for collagen phagocytosis in this system (**figure 2A**). Binding of collagen I to α2β1 triggers the activation of stretch-activated calcium channels [5], leading to an increase in intracellular calcium concentration $[Ca^{++}]_i$. This mediates the accumulation of the actin-binding protein gelsolin and the non-muscular myosin II A (NMMIIA) at the adhesion sites [5]. Gelsolin-dependent nucleation of actin filaments is required for phagosome formation and the internalisation of collagen to early endosomes [6]. Collagen is then delivered to lysosomes, where it is degraded [7]. Furthermore, at least two positive feedback loops reinforce and sustain collagen uptake and degradation. On one hand, $[Ca^{++}]_i$ and the guanine nucleotide exchange factor Vav2 [8] stimulates the small GTPase Rac1 activation, which promotes integrin clustering [9], while, on the other hand, the phosphorylation of NMMIIA by myosin light chain kinase (MLCK) mediates the activation of the small GTPase Rap1, which further sustains collagen binding [10].

Recent pieces of evidence suggest that collagen phagocytosis is strongly dependent on collagen morphology [5], raising the intriguing hypothesis that the complex 3D organisation of the ECM might control its ability to be internalised.

Endo180 mediates clathrin-dependent collagen internalisation

Endo180 (also known as uPARAP) is a member of the mannose receptor (MR) family. It is constitutively internalised to early endosomes (EEs) via clathrin-dependent endocytosis and recycles to the plasma membrane. It has been shown to bind to several collagens, including type I, IV and V, and is required for collagen internalisation and lysosomal delivery [11-13]. Interestingly, the uptake of soluble collagen requires Endo180 but not β1 integrin (**figure 2B**), suggesting that Endo180 mediates a different mechanism from the phagocytic uptake of fibrillar collagen [14]. In line with this, the internalisation of exogenous collagen injected into mouse dermis by fibroblasts does not require Endo180 [15]. A similar Endo180-dependent and integrin-independent pathway has been described in hepatic stellate cells (HSC), which have a key role on controlling ECM homeostasis in the liver [16]. A genome wide RNA interference screen identified Flotillins as important regulators of collagen uptake in monocytes and fibroblasts, through the control of Endo-180, but not α2β1, protein stability [17]. It is not clear what dictates whether collagen is internalised in an α2β1- or Endo180-dependent manner. Several factors, such as cells from different origins and matrix

organisation, may be involved and further studies are needed to clarify this point. Analysis of collagen internalisation by several cell lines highlighted a much higher endocytic rate in mesenchymal cells compared to normal epithelial cells [14], supporting the role of collagen internalisation in connective tissue homeostasis.

β1 integrin mediates caveolin-1-dependent fibronectin internalisation

Fibronectin (FN) is a key component of interstitial ECM and can interact with plasma membrane receptors, including integrins and proteoglycans, as well as with other ECM components, such as collagen and fibrin [18]. FN is secreted by cells as a soluble covalent dimer and polymerised extracellularly to form long fibres [19]. In many cell types, α5β1 integrin is responsible for FN polymerisation, through a process that involves the translocation of α5β1 from focal adhesion to more elongated adhesive structures called fibrillar adhesions [20]. Constant FN polymerisation is required for collagen I deposition and ECM maintenance. Similarly to collagens, FN turnover is mediated by endocytosis and lysosomal degradation. However, this internalisation pathway is dependent on caveolin-1 and not on clathrin, suggesting that different ECM components are endocytosed via different routes [21]. In particular, α5β1 integrin, but not αvβ3, is required for the internalisation of both soluble and matrix FN by myofibroblasts [22]. In migrating fibroblasts, FN binding to α5β1 drives integrin ubiquitination, which promotes the ESCRT-dependent delivery of FN-bound α5β1 to intraluminal vesicles (ILVs) of multivesicular bodies (MVBs), eventually leading to lysosomal degradation [23]. Recent evidence indicates that internalised ECM can also be re-secreted. In epicardial cells, blood vessel epicardial substance (Bves) and N-myc downstream regulated gene 4 (NDRG4) are required for the secretion of internalised FN, by controlling the fusion of recycling endosomes with the plasma membrane (PM) [24]. In cancer cells, endocytosed FN is redeposited at the basolateral surface through a cortactin-dependent pathway regulating the secretion from late endosomes/lysosomes [25]. Finally, the fusion of MVBs with the PM results in the release of FN-containing extracellular vesicles known as exosomes (**figure 2C)**; similarly, cortactin has recently been shown to control collagen I-containing exosome release [26]. It is not clear which mechanisms control FN delivery to different endosomal compartments, resulting in either degradation or secretion.

α3β1 integrin and dystroglycan control laminin internalisation

Laminins (LN) are major components of basement membranes (BM), whose remodelling occurs through proteolytic degradation, suggesting that LN internalisation could be a potential mechanism controlling BM turnover.

Early studies in human breast cancer cells showed that α3β1 integrin, but not α2 integrin, accumulates at the plasma membrane in regions where ECM degradation occurs. Moreover, phagocytosed ECM is trafficked together with α3β1 to acidic vesicular compartments, such as lysosomes, and activation of α3β1 by binding to LN drives phagocytosis of matrigel and gelatin. Interestingly, while α3β1 is required for ECM internalisation, this integrin is not involved in pinocytic uptake of a soluble dye from the media, indicating that α3β1 specifically controls ECM phagocytosis (**figure 2D**) [27]. Despite this, it is possible that receptor-independent macropinocytosis might contribute to LN internalisation. Indeed, such a mechanism has been described for hyaluronan uptake in melanoma cells [28].

The trafficking of LN-111 has been recently shown to be receptor-mediated and dynamin-dependent, leading to lysosomal delivery of LN in normal mammary epithelial cells. Interestingly, LN-111 internalisation does not require integrins, but the LN receptor dystroglycan (DG) (**figure 2D**) [29]. This pathway is compromised in aggressive cancer, where DG is not functional and LN endocytosis is impaired. This suggests that the DG-dependent endocytic trafficking of LN might be required for cells-BM communication in normal epithelial cells, while integrin-dependent LN uptake might be associated with cancer progression.

Cross-talk between extracellular proteolysis and matrix internalisation

The proteolytic cleavage of ECM fibres by MMPs has been suggested to be a pre-requisite for receptor-dependent ECM endocytosis. Indeed, a "collagen breakdown pathway" has been identified in fibroblasts, whereby collagen cleavage by collagenases precede Endo180-dependent uptake [30]. While both intact and cleaved collagen can be internalised, cleaved collagen shows an increased binding to Endo180 and is endocytosed more efficiently. The observation that, even though at a slower rate, intact collagen can be internalised is supported by the fact that, *in vivo*, collagen endocytosis is only partially dependent on MMP activity [15], indicating that proteolytic degradation facilitates but is not absolutely required for ECM uptake. Similarly, internalisation of matrix FN has been reported to be slower than soluble FN and, interestingly, while β1 integrin-null cells are able to uptake soluble FN, the endocytosis of matrix FN is completely impaired [22]. This can be explained considering that clustering of β1 integrin can mediate MMP14 (also known as MT1-MMP) exocytosis [31], leading to localised FN degradation, which is required for α5β1-dependent FN internalisation [32].

Collagen can be degraded by several proteases, and evidence indicates that MMP14 is the main protease involved in collagen endocytosis [30, 33, 34]. In fibroblasts, binding of collagen promotes MMP14 expression, associated with increased collagen degradation and phagocytosis [33]. Moreover, the serine protease Fibroblast Activation Protein (FAP) is responsible for further cleavage of collagen fragments and its activity is required for collagen internalisation [35]. Intriguingly, not only MMP activity can control ECM endocytosis, but also mediators of ECM internalisation have been shown to impinge on MMP activity. Indeed, downregulation of the collagen receptor Endo180 leads to increased membrane targeting and activation of MMP14 [36], supporting the idea of a complex crosstalk between ECM proteolytic degradation and endocytosis. In contrast, MMP activity is not required for Endo180-dependent soluble collagen uptake by lung fibroblasts [37], highlighting that more in-depth analysis of the relationship between matrix organisation and endocytosis is needed.

The role of ECM endocytosis in disease

The balance between ECM deposition and remodelling is crucial for the maintenance of healthy tissue homeostasis. Indeed, several pathological conditions, including fibrosis and cancer, are characterised by aberrant ECM remodelling [2]. In the heart, excess production and deposition of ECM by cardiac fibroblasts results in myocardial stiffness, diastolic and systolic dysfunction [38].

During liver fibrosis, increased activation of fibroblasts and hepatic stellate cells drives both collagen synthesis and degradation, ultimately resulting in net collagen deposition. This represents the major factor causing liver dysfunction. Interestingly, the Endo180-mediated collagen internalisation has

been identified as an important protection mechanism in liver fibrosis *in vivo*, confirming the importance of this degradation pathway in preventing excessive collagen deposition [39]. Similarly, several pieces of evidence link changes in ECM composition and stiffness to cancer initiation and progression [40], suggesting that regulators of ECM remodelling, including ECM endocytosis, could be involved in cancer.

ECM internalisation and cancer

An intact ECM network provides tissue constraint, therefore limiting tumour growth and migration. To overcome this, ECM degradation by MMP release often accompanies cancer progression and promotes metastasis formation. Interestingly, not only MMP-dependent, but also endocytosis-mediated ECM degradation has been shown to impact on cancer invasion and migration. In melanoma cell lines, collagen IV is internalised and delivered to lysosomes, where cathepsin K, a lysosomal cysteine protease, mediates collagen degradation. This process is required for cell invasion [41], suggesting that cathepsin K-mediated intracellular collagen degradation might be required for cancer invasion and metastasis (**figure 3A**).

While in normal mammary glands Endo180 is only expressed by fibroblasts, this collagen receptor is upregulated in a subset of basal-like breast cancers. Similarly to what is observed in fibroblasts, in cancer cells Endo180 also mediates clathrin-dependent collagen endocytosis and lysosomal degradation [11]. Moreover, overexpression of Endo180, but not an endocytosis-deficient mutant, in MCF7 breast cancer cells promotes tumour growth in mouse xenograft experiments, highlighting the contribution of ECM internalisation to cancer formation *in vivo* [42] (**figure 3B**). Consistently, Endo180 levels are increased in metastatic breast cancer patients compared to patients bearing localised (non-invasive) breast cancer [43], suggesting that ECM internalisation might be an important player in the metastatic cascade.

Pancreatic cancer is associated with a very reactive stroma, whereby pancreatic stellate cells (PSC) secrete high amount of collagen. This contributes to cancer cell growth and metastasis. Both pancreatic cancer cells and PSC are able to internalise collagen. Interestingly, the stimulation of epithelial-to-mesenchymal transition (EMT) by transforming growth factor β (TGFβ) strongly stimulates collagen endocytosis by cancer cells, via upregulation of Endo180 [44] (**figure 3C**). Similarly, TGFβ promotes, at least partially, Endo180 expression in glioblastoma cell lines, resulting in increased collagen internalisation and sustained invasion through fibrillar matrices [45].

In ovarian cancer cells, the expression of the small GTPase Rab25 has been associated with increased migration and invasion [46]. Mechanistically, Rab25 drives the internalisation of FN and its delivery to late endosomes and lysosomes. This pathway, which requires α5β1 integrin and its interactor tensin, is required for invasive cell migration and for the activation of mammalian target of Rapamycin (mTOR), a key regulator of nutrient signalling [47], suggesting a link between ECM internalisation and cancer cell metabolism (**figure 3D**). Consistent with this, it has been hypothesised that collagen-rich ECM might represent a proline-reservoir: upon collagen cleavage by MMPs, its degradation sustains proline metabolism in pancreatic tumours [48]. Therefore, the ECM could be considered as a source of nutrients to sustain cancer growth.

Exosome secretion has a key role in controlling cell-cell communication, as well as cancer invasion and metastasis. Several ECM components have been identified as exosome cargos and FN is required

for the exosome-dependent migratory phenotype in fibrosarcoma cells (**figure 3E**) [49]. Interestingly, a positive feedback loop has been identified in breast cancer cells between invadopodia formation and exosome secretion, which sustains cancer cell invasion [50]. Although the exosome cargos responsible for this have not been described, one intriguing hypothesis is that ECM degradation at invadopodia leads to the generation of ECM fragments, available to be internalised, delivered to exosomes and secreted, to locally promote invasiveness.

Concluding remarks

ECM internalisation is an important mechanism through which ECM remodelling is controlled. It is now clear that the ECM is not a passive by-stander but is actively involved in maintaining tissue homeostasis. In pathological conditions, such as in cancer, the complex interplay between cells and the ECM provides the right environment to foster cancer growth and migration. It is therefore extremely important to deepen our understanding on how ECM endocytosis is regulated, with the ultimate aim of developing novel therapeutic strategies based on this process. For this to happen, it is essential that the findings obtained from cell lines in 2 dimensional (2D) settings are implemented using more physiological 3D environments, such as the use of cell-derived matrices [51] and 3D culture systems. It will also be important to assess how matrix composition and 3D organisation affect its ability to be endocytosed. Moreover, endocytosis is intimately linked with modulation of receptor signalling. Similarly to what observed with receptor tyrosine kinases, it can be expected that, upon ECM binding and internalisation, different signalling complexes could be assembled at the plasma membrane and along the endocytic pathway. Indeed, recent evidence indicates that integrins can signal from endosomes and this is important for anchorage-independent cancer cell proliferation [52]. Therefore, it will be important to investigate how ECM endocytosis contributes to and cooperates with oncogenic signalling during cancer progression.

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Figure legends

Figure 1: schematic representation of ECM organisation. The basement membrane is mainly composed of collagen IV, laminins, perlecan, agrin and nidogen; while the interstitial matrix contains collagen I, fibronectin, tenascin C and elastin. Adapted from [2].

Figure 2. Schematic representation of the endocytic pathways involved in ECM internalisation. Collagen can be internalised though an α2β1 integrin-dependent phagocytic process (**A**) or via Endo180- and clathrin-dependent endocytosis (**B**). Fibronectin internalisation occurs via a α5β1-, caveolin- or ESCRT-dependent pathway (**C**); while two laminin (LN) receptors, dystroglycan (DG) and α3β1 integrin mediate LN internalisation (**D**). These pathways can lead to the delivery of the ECM components to early endosomes (EEs) and lysosomes (Lys), where the ECM is degraded. Alternatively, internalised ECM can be re-secreted via Lys fusion or multivesicular body (MVB)-dependent release of intraluminal vesicles (ILVs).

Figure 3. Schematic representation of the contribution of ECM uptake to cancer phenotypes. Cathepsin K (catK)-mediated collagen degradation in the lysosomes (Lys) promotes melanoma cell invasion (**A**). Endo180-dependent collagen internalisation mediates breast cancer growth in mouse xenograft experiments (**B**). In pancreatic cancer cells, collagen internalisation is upregulated during epithelial to mesenchymal transition (EMT), via increased Endo180 expression induced by transforming growth factor β (TGFβ) (**C**). α5β1 integrin controls fibronectin endocytosis in ovarian cancer cells, mediating invasive cell migration and mTOR signalling (**D**). The secretion of ECM-containing exosomes controls cancer cell migration and invasion (**E**).

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Figure 1

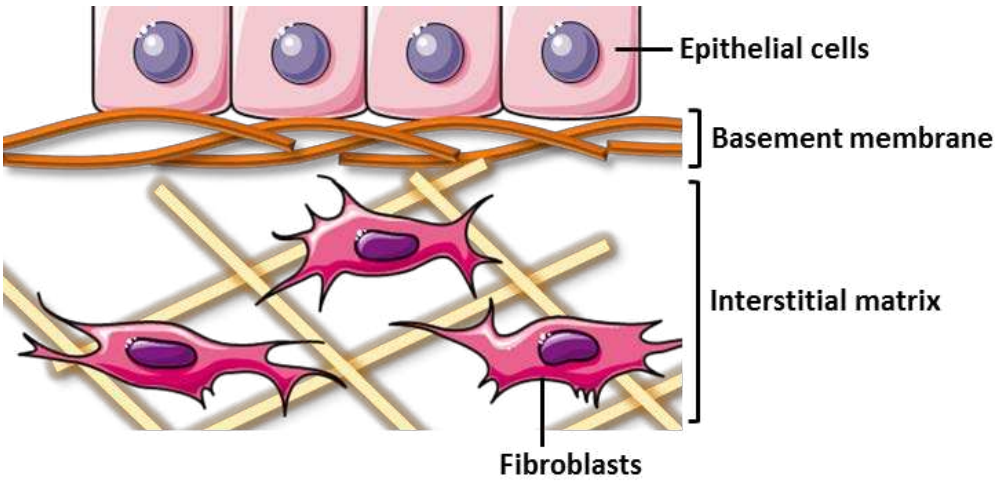
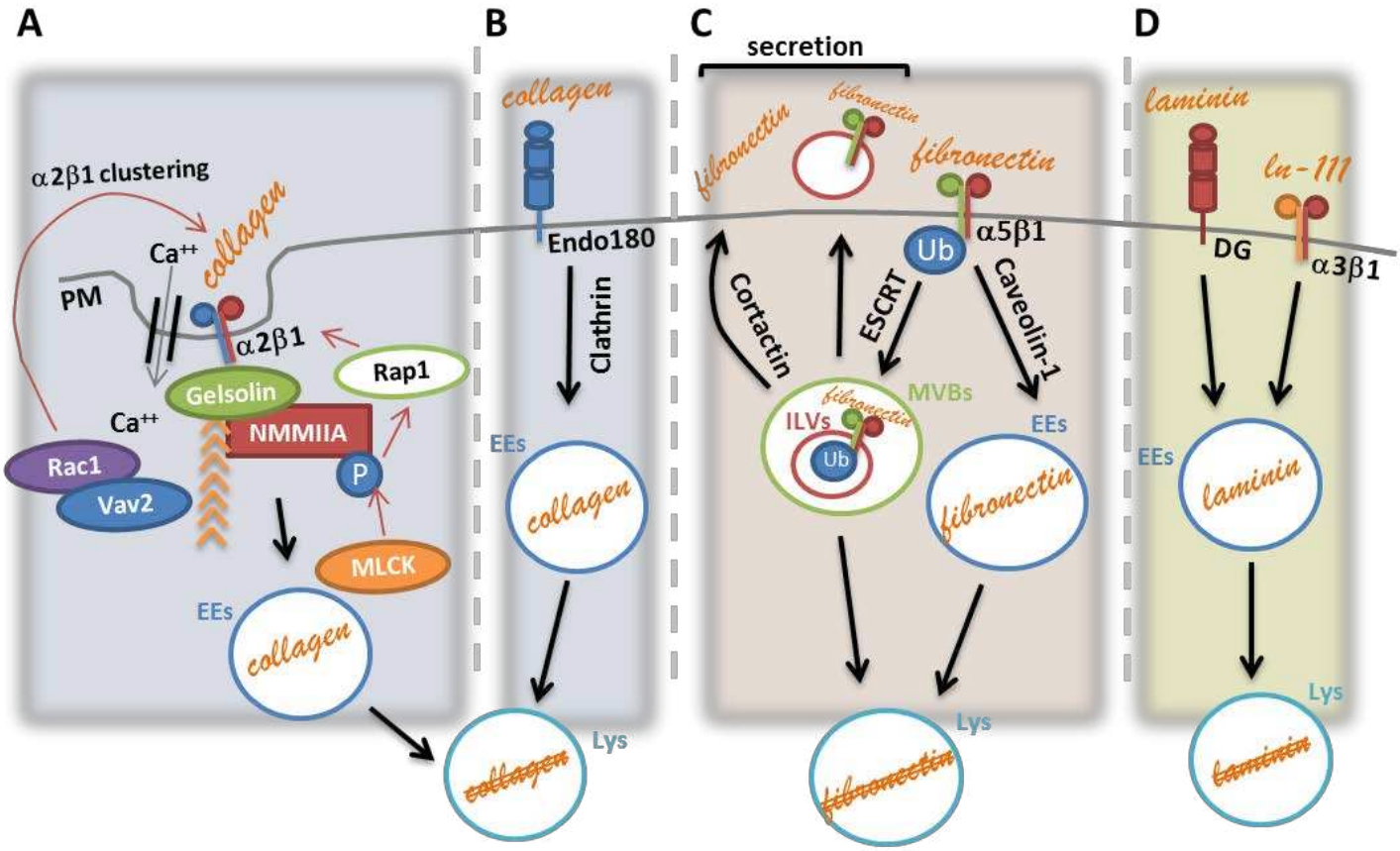

Epithelial cells
Basement membrane
Interstitial matrix
Fibroblasts

Figure 2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Ca++
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P
MLCK
EEs
collagen
collagen
Lys
collagen
Endo180
Clathrin
EEs
collagen
secretion
fibronectin
fibronectin
fibronectin
α5β1
Ub
Cortactin
ESCRT
Caveolin-1
MVBs
ILVs
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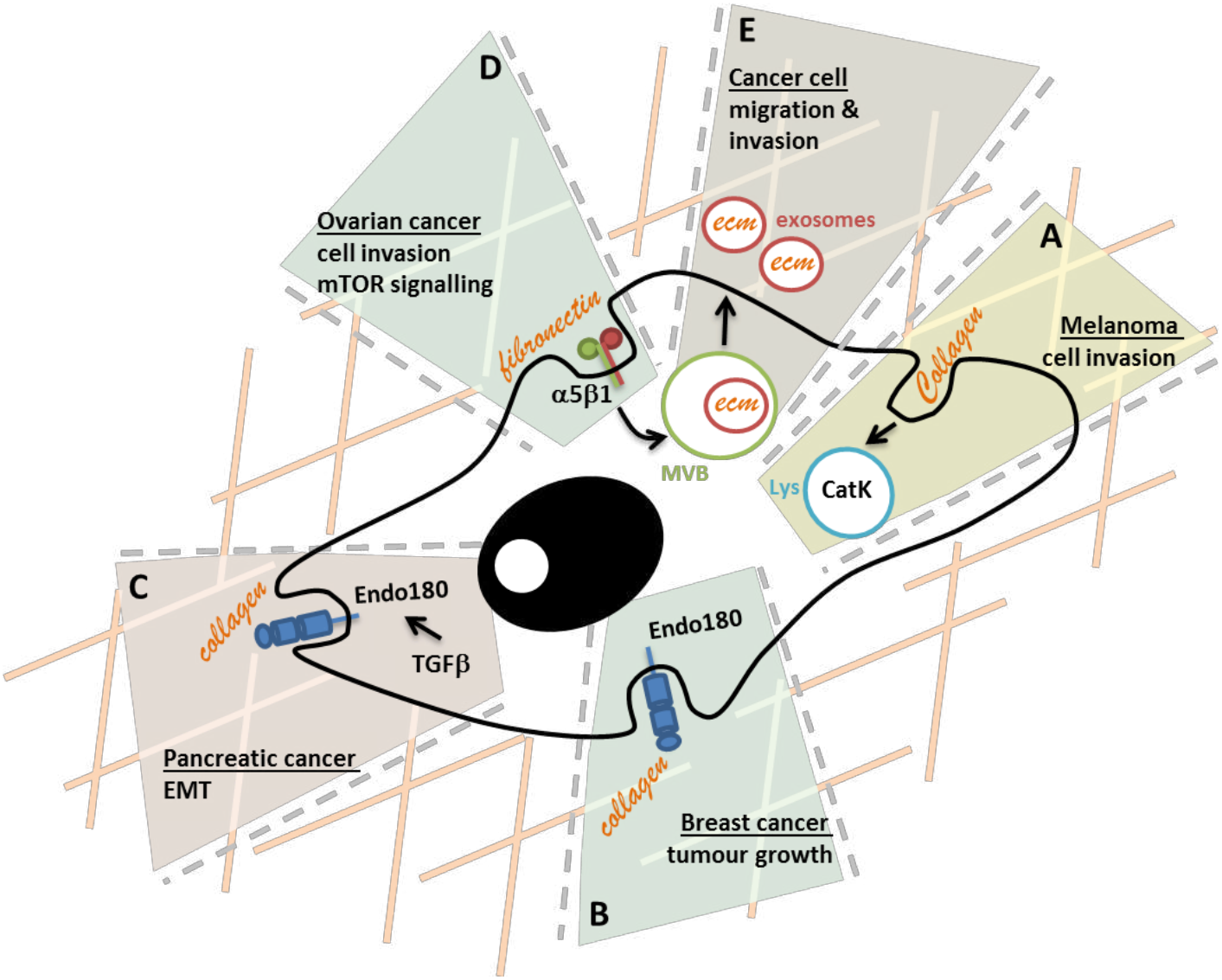
laminin

Figure 3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Melanoma
cell invasion
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Lys
CatK
C
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Endo180
TGFβ
Pancreatic cancer
EMT
Endo180
collagen
Breast cancer
tumour growth
B