Ann Ist Super Sanità 2010 | Vol. 46, No. 1: 66-80
DOI: 10.4415/ANN_10_01_09

Molecular aspects of tumor cell migration and invasion

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Summary. Cell migration and invasion are crucial steps in many physiological events. However, they are also implicated in the physiopathology of many diseases, such as cancer. To spread through the tissues, tumor cells use mechanisms that involve several molecular actors: adhesion receptor families, receptor tyrosine kinases, cytoskeleton proteins, adapter and signalling proteins interplay in a complex scenario. The balance of cellular signals for proliferation and survival responses also regulates migratory behaviours of tumor cells. To complicate the scene of crime drug resistance players can interfere thus worsening this delicate situation. The complete understanding of this molecular jungle is an impossible mission: some molecular aspects are reviewed in this paper.

Key words: neoplasms, neoplasm invasion, molecular markers.

Riassunto *(Aspetti molecolari della migrazione ed invasione delle cellule tumorali)*. La migrazione e l'invasione cellulare rappresentano momenti cruciali in molti eventi fisiologici: questi due processi, tuttavia, sono anche implicati nella fisiopatologia di varie malattie, tra cui i tumori. Per diffondersi attraverso i tessuti, le cellule tumorali ricorrono a meccanismi che vedono il coinvolgimento di diversi componenti cellulari: famiglie di molecole di adesione, recettori tirosinchinasi, proteine del citoscheletro, proteine di segnalazione intracellulare intervengono in un complesso scenario molecolare. Le vie di segnalazione regolanti i processi di sopravvivenza e proliferazione cellulare giocano un ruolo importante anche nei comportamenti migratori delle cellule tumorali. A complicare la scena del crimine, marcatori proteici della farmacoresistenza contribuiscono al conferimento di un fenotipo maggiormente aggressivo, peggiorando in tal modo una situazione già di per sé delicata. La comprensione completa di questa "giungla molecolare" è una missione impossibile: in questa rassegna verranno presi in considerazione alcuni degli aspetti molecolari.

Parole chiave: neoplasie, invasione delle cellule tumorali, marcatori molecolari.

INTRODUCTION

Cell migration and invasion are crucial steps in many physiological events such as implantation of embryo, embryogenesis, morphogenesis, neurogenesis, angiogenesis, wound healing and inflammation [1, 2]. However, cell migration and invasion are also implicated in the pathophysiology of many diseases, such as cancer. Indeed, the capacity to produce metastases, very different among cancers, is the main features of malignant tumors and it is one of the main causes of death for cancer. This is due to the fact that metastases are constituted by cells much more resistant, aggressive and efficient than those forming the primary tumor.

In the last years, major efforts have been undertaken to understand the molecular mechanism underlying the distinct steps of metastasis, which are (i) detachment of tumor cells from the primary tumor, (ii) invasion into surrounding tissue, (iii) intravasation into blood or lymphatic vessels, (iv) dissemination in the blood stream or the lymphatic system and, finally, (v) extravasation and outgrowth at a secondary site. Each of these steps requires a distinct molecular program in which the modulation of the adhesive and migratory as well as the cytoskeletal properties of the disseminating tumor cells play essential roles.

Tumors can spread in a variety of channels/ways: the most common pathway is tumor extending in continuity beyond the organ or structure of origin, *i.e.* when it passes from the original organ to another organ or vessel or cavity by continuity. Dissemination for contiguity occurs when tumor infiltrates tissue spaces of non continuous adjacent structures.

The most common pathways for distant spread in the chest and abdomen are the lymphatics, blood vessels, and coelomic cavities. Cancer cells can disseminate from the primary site via lymphatic routes ("lymphatic metastases") and by haematogenous routes ("*ab initio* hematogeneous metastases"). Secondary haematogenous dissemination of lymphatic metastases also occurs from overt metastases to other distant sites. Coelomic cavities involved in tumor dissemination include the pleural space of the thoracic cavity and the peritoneal spaces of the ab-

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domen and pelvis. The most commonly involved is the peritoneum, which carries tumor cells in ascitic fluid. The distribution of intraperitoneal metastases often corresponds to predictable flow patterns, the most classic example of which is seen with ovarian cancer. With this tumor, or any tumor demonstrating intraperitoneal spread, the paracolic gutters, cul-de-sac, omentum, and liver surface are common sites of metastases. In the chest, pleural dissemination typically spreads via gravitational forces and is often seen in the lower thoracic cavity [3].

Each phase involved in the metastatic process requires many specific molecular interactions between the tumor cells, the extracellular matrix (ECM) and the cells of the stroma. Liotta *et al.* [4] proposed the well-known three-steps theory for tumor cell invasion: in the first step tumor cell adhere to specific components of the matrix through cell surface receptors; in the second step, the anchored tumor cell secrets hydrolytic enzymes which can locally degrade the matrix. The third step is represented by the tumor cell migration through the matrix region modified by proteolysis.

To spread through the tissues, tumor cells use mechanisms that are similar but not identical to those used by normal cells during physiological processes such as morphogenesis and migration of immune cells [5]. Cell migration was firstly studied in fibroblasts, keratinocytes and myoblasts [6, 7]. Further studies showed that some basic strategies are also preserved in tumor cells.

The cell migration through the tissues results from a continuous cycle of coordinated and interdependent steps that involve the cytoskeletal machinery [7, 5]. Migration begins when a cell responds to an external signal that leads to the polarization and extension of a "leading front" in the direction of the movement. Then, the leading front binds to ECM proteins, and the cell body shrinks: a traction force is thus generated, which determines the slow sliding of the cell body behind the migrating front.

In the first phase of the migration process the extension of cell protrusions is driven by actin polymerization. This reaction may be increased by the actin monomer addition to existing filaments or by the nucleation of new actin filaments by Arp2/3 (actin-related protein 2/3) [8]. Arp2/3 is predominantly regulated by the family of adapter proteins WASP (Wilscott-Aldrich syndrome protein) and WAVE (WASP-family verprolin-homologous protein), which act as a molecular platform for the formation of the complex responsible for actin nucleation. As described in the speculative model proposed by Rohatgi *et al.*, [9] Cdc42 and lipids (PI(4,5)P2) may regulate actin assembly at membrane-proximal sites by recruitment and activation of the Arp2/3 complex via N-WASP-like proteins. Phosphoinositides bind and activate the guanosine-nucleotide-exchange factors (GEFs) that, in turn, regulate the activity of small GTPases [10] able to activate actin assembly regulatory proteins.

The cellular protrusions that initially recognize and bind to the ECM may be morphologically different: lamellipodia, filopodia, pseudopodia and invadopodia [11], all contain filamentous actin. Indeed, the propulsion and elongation of pseudopodia are driven by actin polymerization and by filament assembly [9, 12]. Growing cell protrusions reach and begin to bind to adjacent ECM by means of adhesion molecules such as integrins and cadherins: these molecules are involved in the formation of "focal contacts" complex composed of extracellular ligands, tyrosine kinase receptors and cytoskeletal proteins.

ADHESION MOLECULES

The high degree of specificity that characterizes both the cell recognition and the adhesion phenomena requires the interaction between molecules to constitute cell-cell or a ECM-cell bridges. These proteins are the so-named "adhesion molecules" or CAMs (cell-cell adhesion molecules), glycoproteins expressed on the cell surface. Most of CAMs belongs to six protein families: cadherins, integrins, immunoglobulin superfamily, selectins, lymphocyte homing receptors. CAMs are involved in many physiological and pathological processes, and it is now well known that they can assume a key role in the complex evolution of metastases [13].

Cadherins (calcium-dependent adhesion molecules) belong to a large family of transmembrane glycoproteins that mediate cell-cell adhesions in a calcium-dependent manner [14]. The epithelial cadherin (E-cadherin) has been the first adhesion molecules to be discovered and characterized. Thus, E-cadherin is the prototype member of cadherin family and plays a fundamental role in the development and maintenance of adhesion between epithelial cells [15]. E-cadherin consists of an extracellular domain, constituted of five cadherin repeats (EC1, EC2, EC3, EC4 and EC5), a transmembrane domain, and an intracellular domain that binds to both P120 catenin and beta-catenin. It has been well documented that tumors of epithelial origin partially, or totally, lose the expression of E-cadherin with the acquisition of a more aggressive phenotype [16]. On the other hand, several studies have also shown the strong anti-invasive and anti-metastatic role of E-cadherin [17-19].

The large family of integrins comprises a wide number of cellular receptors, heterodimeric transmembrane glycoproteins constituted of two subunits, the alfa chains associated with the beta chains through non-covalent bond [20]. Integrins are adhesion molecules essential in the intercellular interactions and in the integration (hence the name) of cells with the extracellular environment. Both alfa and beta chains penetrate into the cell membrane giving rise to the cytoplasmic domains essential for signal transduction. The molecular mass of alfa subunits varies between 120 and 180 kDa, whereas that of the

beta subunits ranges from 90 to110 kDa. By different combinations of 18 alfa chains and 8 beta chains are generated 24 distinct integrins [21]. Outside of the plasma membrane the alfa and beta subunits protrude about 23 nm, and the NH2 terminal ends of each chain are used to link the ECM. The main role of these adhesion molecules, in fact, is to mediate cell-matrix and cell-cell interactions [22].

The integrins are involved in many physiological and pathological processes, including inflammation and wound repair [23], proliferation, differentiation and apoptosis [24]. In particular, they seem to have a crucial role in metastasis, by mediating the interaction of tumor cells with the ECM [25]. These roles are possible thanks to the physical bond of adhesive contacts with the actin cytoskeleton, with the consequent activation of cytoplasmic pathways mediated by different signal proteins such as Rho, Src, MAPKs and PKB [22]. Evidence of the connections with the cytoskeleton comes from a large number of studies conducted by electron microscopy and demonstrating the co-localization of integrins with the cytoskeletal structures [26]. The integrins bind to a wide range of matrix proteins, including laminin, fibronectin, trombospondin, vitronectin and various types of collagen [27-29].

A widely used classification of integrin superfamily is based on the type of chain constituting the heterodimer. VLAs are integrins belonging to the beta1 subfamily, and consist of six heterodimers. They are called "very late activation antigens" (VLAs) because the first glycoproteins identified (VLA-1 and VLA-2) were only expressed at a late stage after T-cell activation. The beta1 integrins are expressed on lymphocytes [20, 30], where they mediate the binding with the proteins of the ECM, playing an important role in the extravasation and migration in tissues during the immune response [31]. In this subfamily the beta1 subunit binds to 6 different alfa subunits, leading to the integrin classification VLA1, VLA2, VLA3, VLA4, VLA5, VLA6. VLA2 consists of the alfa 2 and beta 1 subunits, binds to different types of collagen (I-II-III-IV) and to laminin I. VLA5 (alfa5, beta1) binds to fibronectin and to the adhesion molecule L1CAM (L1 cell adhesion molecule).

Another adhesion molecule widely expressed on lymphocytes is CD44. This small molecule is a membrane glycoprotein of Class I of 85-95 kDa. CD44 is encoded by a single gene [32] but the heterogeneity of produced protein is partly generated by post-transcriptional modifications [33], that differ with respect to both cell type and growth conditions. This glycoprotein is able to bind to laminin, fibronectin, collagen and, particularly, to hyaluronic acid, an important ECM component [34]. The CD44 is a multifunctional receptor, not only important in the context of the immunological response. Similar to integrins discussed above, CD44 was initially detected on the membrane of the immune system cells. Its identification on other type of cells has expanded its function [35]. Gilcrease *et al.* [23] demonstrated that a high expression of VLA2 and CD44 was associated with a high capability of producing metastases by renal carcinoma. In fact, by analyzing the adhesion molecule expression in 37 cell lines, isolated from nephrectomies, and the relative behaviour in the extra-renal stroma, a positive correlation was observed between invasive capacity and level of expression of both the molecules on the cell membrane. A study published in 2006 also highlighted the correlation between VLA2, VLA5 and CD44 adhesion molecules, and tumor metastases in human osteosarcoma cells [36]. Very important is also the association of CD44 with ezrin, radixin, myosin (ERM) and merlin (moesin-ezrin-radixin-like protein) proteins [37-39]. In particular, ERM proteins are essential for the regulation of protein movements in the plasma membrane, cells shape and cell migration [40, 41].

INTERACTION WITH THE ECM AND FORMATION OF FOCAL CONTACTS

The integrin family of heterodymeric transmembrane receptors play a particularly important role in the interaction with ECM and formation of "focal contacts" [24, 42]. Cells adhere to ECM via integrin-mediated adhesions that link matrix to actin cytoskeleton. In cultured cells, integrin-based molecular complexes form discrete morphological entities of several types. Small (0.5-1 μm) "dot-like" or "point contacts" also known as "focal complexes" are localized at the edges of lamellipodia. Elongated (3-10 μm in length) streak-like structures associated with actin- and myosin-containing filament bundles (stress fibers) are known as "focal contacts" or "focal adhesions" [43]. "Podosomes" and "invadopodia" are highly dynamic and specialized adhesive structures, rich in focal contacts. They contribute to remodel the cytoskeleton and the matrix by controlling both the local turnover of focal contacts and the degradation of ECM.

Following the contact with specific ECM ligands, integrins clusterize on the cell membrane, and recruit through their intracellular domain either adapter proteins or signal proteins. This leads to phosphorylation and dephosphorylation signals within the cell. In particular, cytoplasmic region of integrins directly interacts with adapter proteins such as alpha actinin, tensin, talin and the signal protein FAK (focal adhesion kinase). All these proteins can in turn bind to other adapter proteins to recruit in focal contacts actin ligands, such as vinculin, paxillin and alpha-actinin, which are all involved in the dynamic association with actin filaments. [43, 44].

Assembly of focal contacts is regulated through various signaling pathways that include the phosphatidylinositol 3-phosphate (PI3K), the protein kinase C (PKC) and Rho family GTPases [44, 45].

The dynamics of focal cell-ECM adhesions is determined by the cyclic formation and destruction of these structures, and both intracellular calpain pro-

teinases and the ubiquitin-dependent proteasome system are involved in these regulatory mechanisms [46-48].

Focal adhesions allow the cell to acquire a "morphological polarization", which results in a directional motility. The generation of a protrusive force, produced by the asymmetric actin polymerization on the leading edge of the cell, and the generation of a contractility force within the cell, through the interaction between actin filaments and myosin motor system, contribute to the process of directional migration. In some cases, during the processes of migration and invasion, tumor cells are able to adopt an "ameboid" mechanism of propulsion, involving integrin-independent adhesions and actomyosin-dependent expansion/contraction cycles. These "ameboid" cells circumnavigate, rather than degrade, the ECM physical barrier [49]. In other cases, tumor cells actively invade different tissues through a turnover of adhesion molecules that allow the cell to form contractility structures and degradate the ECM [50].

CELL CONTRACTION: REGULATION OF ACTIN-MYOSIN COMPLEX

Actin-myosin contraction promotes the cell shortening along the long axis, and generate internal tension in the direction of the focal contacts located on the leading edge. Subsequently, the cell-substrate bonds preferentially dissolve at the rear edge of the cell, while the migration front, still attached, moves further forward [51, 52]. This induces the slow sliding of the cell rear edge forward. Organization, assembly and the tension of the actin-myosin skeleton are controlled by different regulatory enzyme systems. One of such systems directly controls the myosin contractility, by modulating the light chain (myosin light chain, MLC) through the MLC kinase activity (MLCK) and MLC phosphatase (MLCP) counteraction. The activity of these enzymes, in turn, is regulated by another set of enzymes, the Rho-GTPases, belonging to the group of small GTPases (guanosine triphosphatases). This group includes several members: Rho, Rac and CDC42 proteins [53-55].

Targets of Rho protein are mDia1 (mammalian Diaphanous 1), LIMKs (LIM kinases), and Rho-associated kinase (ROCK). mDia1 is a mammalian homolog of *Drosophila diaphanous* and works as an effector of the small GTPase Rho. LIM kinase-1 (LIMK1) and LIM kinase-2 (LIMK2) are actin-binding kinases that phosphorylate members of the ADF/cofilin family of actin binding and filament severing proteins. Rho-associated kinase (Rho-kinase/ROCK/ROK) is a serine/threonine kinase and plays an important role in various cellular functions.

ROCK phosphorylates and inhibits the activity of MLCP: this inhibition in turn results in increased MLC phosphorylation and, consequently, in an increase of the actin-myosin complex contractility. ROCK in cooperation with mDia1 can also stimulate the formation of "stress fibers". ROCK phosphorylates and activates LIMK1 which in turn inhibits the depolymerisation of actin microfilaments [56]. Finally, mDia1 promotes actin polymerization and microtubule stabilisation. It has been suggested that mDia1 also increases the rate of cytoskeletal reorganization [57].

The increase of contractile tension induced by Rho results in the formation of numerous actin bundles associated with mature focal contacts. In contrast, ROCK and Rho inhibition leads to actin disassembly and focal contacts processing in small focal complexes: this transformation is probably the consequence of the decrease in actin-myosin tension [56].

Rho and MLCK allow cells to separately control the movements of cortical actin from the contraction of actin filaments, located in the deeper layers of the cytoplasm. Indeed, the contraction of actin filaments, controlled by myosin II, is induced primarily by Rho and its effector ROCK [58, 59]. In contrast, the cortical actin network seems to be regulated by MLCK and not by Rho [60, 61].

Therefore, the Rho protein family plays a central role in regulating the movement of the cytoskeleton: the level of action of these proteins is regulated by the balance between a state of activation mediated by the GEFs exchange factors (GTPase exchange factors), which facilitate the replacement of GDP with GTP, and a state of inactivation where GAPs (GTPase-activating proteins) operate by attenuating the signal through the stimulated hydrolysis of bound GTP [62].

Besides Rho, other proteins of the same group, Rac and CDC42, can play an important role in regulating actin movements in migrating cells. Activated Rac stimulates the "ruffling" of the membrane, which results in the formation of the actin-rich cellular protrusions lamellipodia, while activated CDC42 stimulates the actin polymerization and the formation of the thin membrane protrusions filopodia [62]. The activity of Rho GTPase also regulates other types of dynamic structures such as cadherin-dependent intercellular junctions, thus mediating the transition from epithelial to mesenchymal phenotype. The epithelial-mesenchymal transition (EMT) is a frequent event in the progression of various malignancies and is characterized by loss of expression of E-cadherin and acquisition of expression of N-cadherin and vimentin.

THE INVADOPODIA

The invadopodia are cellular protrusions rich in actin that can mediate the proteolysis of ECM [63-65]. The molecular origin and the role of invadopodia in particularly aggressive tumors such as gliomas, breast carcinomas and melanomas have been recently discussed [63]. These subcellular organelles have been observed for the first time in fibroblasts,

genetically modified by inserting v-Src oncogene, which encodes for a protein with tyrosine kinase activity. Following transformation, this protein is constitutively activated and cells, grown on fibronectin, form prominent protrusions with both adhesive and degradation properties [66, 67]. The invadopodia have a diameter varying between 0.1 and 0.8 μm and their length can be up to 2 μm. Several markers are used for their identification: F-actin (filamentous actin form), Arp2/3, N-WASP and cortactin, SH3-proteins involved in various tumors [69-73].

The maturation of invadopodia sees a four-steps process: (i) location of cortactin on the site of the protrusion formation; (ii) recruitment of MT1-MMP metalloproteinase (membrane-type type 1 MMP); (iii) degradation of the matrix; (iv) dissociation of the cortactin from the cell membrane [70].

Several proteins are involved in the molecular pathways responsible for the invadopodia formation, whose invasive properties are attributed mainly to integrin-ligand binding *(Figure 1)* [69]. The molecular cascade starts with the ECM-integrin linkage, together with the interaction of growth factors, such as the "epidermal growth factor" (EGF) and the "fibroblast growth factor" (FGF), with their receptors. The recruitment and the activation of other components occur downstream, outlining a complex molecular network in which many pathways are still unknown. Src is the first cytosolic protein involved, and it is activated by phosphorylation. Following the activation mediated by activated Src, cortactin binds to N-WASP, F-actin and Arp2/3 (these two molecules are also triggered by activated N-WASP), and inhibits actin depolimerization. This cooperative action is essential to allow the actin assembly, physiological process essential in invadopodia formation. Experiments with RNA interference, negative mutants or inhibitory antibodies show that proteins such as cortactin [70, 72, 74], N-WASP [73, 75], AMAP1/ASAP1 (protein belonging to Arf GAP family, and complexed with a peptidase acting as GTPase) [76] and Tks5/Fish (an anchor protein with SH3-domains) [77] are necessary for the degradation of ECM mediated by the invadopodia in various tumor cell lines. Tks5/Fish, thanks to its anchoring domains, binds to N-WASP, whereas AMAP1/ASAP1 binds to cortactin: these associations are important for the subsequent formation of invadopodia.

FOCUSED PROTEOLYSIS OF ECM: THE METALLOPROTEINASES

Degradation and remodeling of ECM are essential stages of migration, invasion and metastasis of cancer cells. These processes are primarily mediated by two types of proteolytic enzymes: the plasminogen activator system components and the matrix metalloproteinases (MMPs) [78].

The MMPs belong to a family of zinc-dependent endopeptidases, highly conserved and structurally related, capable of degrading many components of basement membrane and ECM [79]. The substrates of MMPs include a wide variety of proteins such as chemotactic molecules, adhesion molecules, proteinase inhibitors, cell surface receptors, blood coagulation factors, growth factors and growth factor-binding proteins. Studies on the activity of MMPs in various cells and tissues, demonstrated the importance of these enzymes in many physiological (*e.g.* embryonic development, bone resorption, angiogenesis) and pathological processes (*e.g.* rheumatoid arthritis, multiple sclerosis, tumor growth and metastasis) [80, 81].

Human MMPs generally contain a signal peptide, a N-terminal pro-peptide domain, a catalytic domain that includes highly conserved zinc-binding sites, and a hinge region followed by an hemopexin-like C-terminal domain *(Figure 2a)*.

They can be divided into different classes according to their sequence homology, substrate specificity, cellular localization and structure *(Figure 2b)*. MMP-2 and MMP-9 gelatinases present an additional domain inserted between the catalytic domain and the active site domain. MT-MMPs may have an additional transmembrane domain site, either a glycosylphosphatidylinositol anchor site (GPI) or a Ig-like domain that determines the localization on the cell surface.

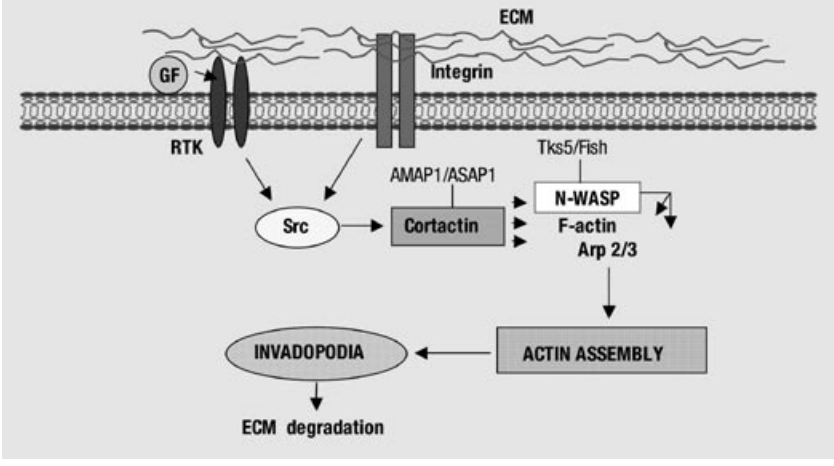

Fig. 1 | *Molecular pathway underlying invadopodia formation and ECM degradation. GF and RTK indicate the growth factor and the relative receptor tyrosine kinase, respectively. Lines indicate an association between components; arrows an activation sequence.*

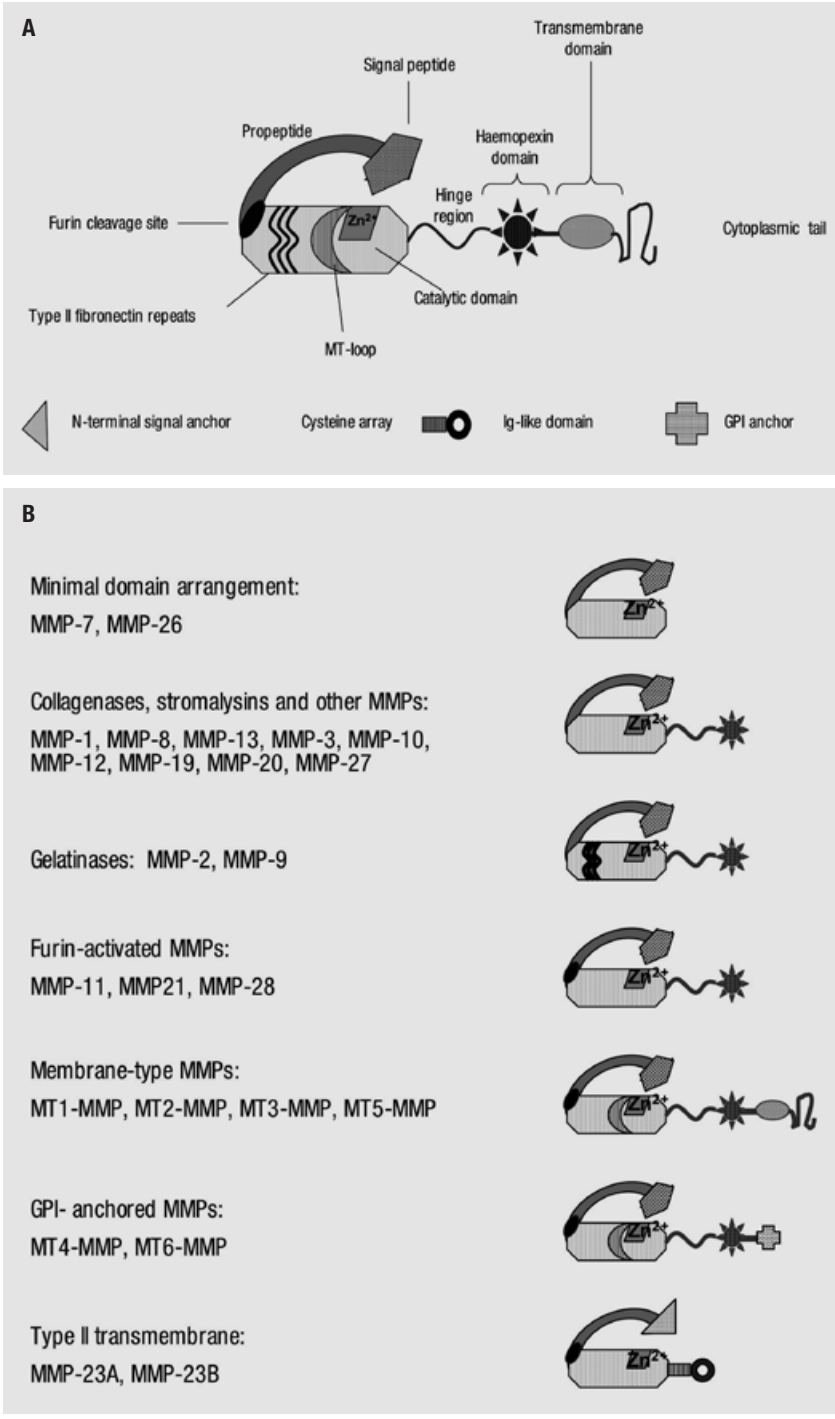

Fig. 2 | *a) General structure of metalloproteinases. b) Classification of MMPs according to their structure. (Modified from: Lafleur MA, Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in the angiogenesis.* Expert Rev Mol Med *2003;5:1-39).*
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According to substrate five MMP subclasses have been defined: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromalysins (MMP-3, MMP-10, MMP-11), metalloelastases (MMP-12, MMP-18, MMP-19), matrilysins (MMP-7), membrane-type-MMPs (MT-MMPs) [82, 83].

The expression, secretion, and activity of MMPs are finely controlled in normal tissue. In particular, the expression of MMPs is regulated at both transcriptional and post-transcriptional level. Several factors influence the transcription of genes coding for these endopeptidases, including cytokines, growth factors, hormones, oncogenes and tumor promoters [84, 85]. Cytokines and growth factors are able to regulate the expression of metalloproteinases through the MAPKs pathway that includes proteins such as ERK 1/2 (extracellular regulated kinase 1/2), JNK/SAPK1/2 (c-Jun N-terminal kinase 1/2) and p38MAPK. Thus, the activation of AP-1 and ETS transcription factors by the mitogen kinases is responsible of the maximum expression of MMPs [85].

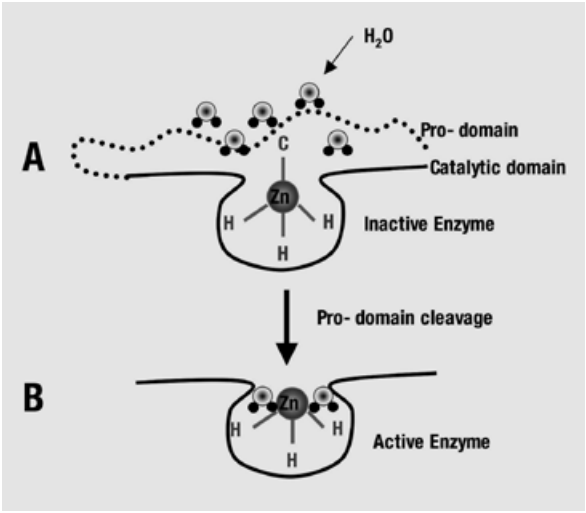

Fig. 3 | *"Cysteine switch" activation mechanism. a) A conserved cysteine-rich domain, located in the pro-domain, forms a bond with the coordinated zinc ion located in the active site. b) The cleavage of the pro-domain leads to the breaking of zinc-cysteine bond followed by the amino-terminal pro-domain loss (Modified from: Somerville RPT, Oblander SA, Apte SS. Matrix metalloproteinases: old dox with new tricks.* Genome Biol *2003;4:216-26).*
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At post-transcriptional level, the biological activity of MMPs is regulated by their state of activation. Many MMPs are secreted from cells in latent form as zymogens (pro-MMPs). The pro-MMPs conversion in a functionally active form requires a more specific multi-stage process known as "cysteine switch" and that leads to the proteolytic removal of parts of the molecule *(Figure 3)*. A highly conserved cysteine-rich domain, located in the prodomain, links to zinc ion in the active site *(Figure 3a)*. The prodomain cleavage leads to the rupture of the zinc-cysteine (Zn-C) bond, followed by the amino-terminal prodomain loss *(Figure 3b)*: in this way the active site becomes accessible. For many MMPs proteolytic activation begins in the extracellular space by serine proteases, or by other members of the family of MMPs [86]. On the cell surface, the MT-MMPs (membrane-type MMPs) have been identified as potent physiological activators of several MMPs [82, 87].

The activity of MMPs is modulated by the family of "tissue inhibitors of metalloproteinases" (TIMPs) that are able to inhibit active MMPs after binding in their catalytic domain. In addition, TIMP-1 and TIMP-2 regulate the activation of some pro-MMPs by binding to their carboxy-terminal domains. In particular, TIMP-1 inhibits the activation of pro-MMP-9, while TIMP-2 binds and regulates the activation of pro-MMP-2 [88]. As shown in *Figure 4*, MT1-MMP can form a complex with TIMP-2, which subsequently serve as a receptor for pro-MMP-2. A second molecule of MT1-MMP adjacent to the complex, not inhibitor-linked can convert pro-MMP-2 in its active conformation [79, 89]. In particular, at low concentrations, TIMP-2 promotes the formation of a complex with pro-MMP-2 and MT1-MMP on the cell surface, thus leading to activation of MMP-2 *(Figure 4a)*. However, at high concentrations TIMP-2 inhibits the activation *(Figure 4b)*.

The active MMPs may remain localized on the cell surface through binding with membrane molecules, and this leads to a more focused ECM degradation. The expression of MMPs and TIMPs changes during the neoplastic transformation. A high secretion of MMPs by tumor cells has been demonstrated in many types of cancer [3, 90] and the imbalance between MMPs and their specific inhibitors seem to play an important role in the tumor growth and invasion [3, 78].

ROLE OF MITOGEN-ACTIVATED PROTEIN KINASES (MAPKS) IN THE PROCESSES OF CELL MIGRATION AND INVASION

Many extracellular signals converge on the pathway of proteins belonging to the serine/threonine

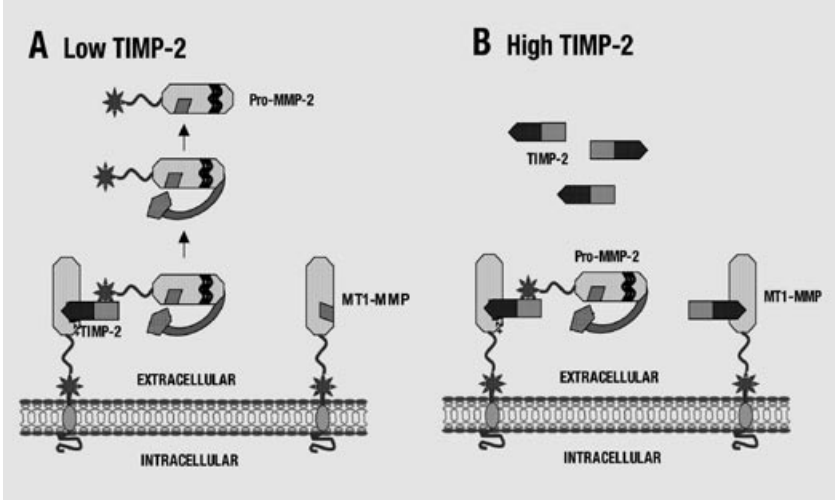

Fig. 4 | *Mechanism of activation of pro-metalloproteinase 2 (pro-MMP-2). a) When present at low concentrations, TIMP-2 can form a complex with a molecule of MT1-MMP, which in turn will serve as a receptor for proMMP-2. A second molecule of unbound MT1-MMP, adjacent to the complex, can convert proMMP-2 in its active conformation. b) At high concentrations, TIMP-2 forms a complex with both proMMP-2 molecules, already bound to MT1-MMP, and with MT1-MMP free molecules thus inhibiting the activation of MMP-2 (Modified from: Lafleur MA, Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in the angiogenesis.* Expert Rev Mol Med *2003;5:1-39).*
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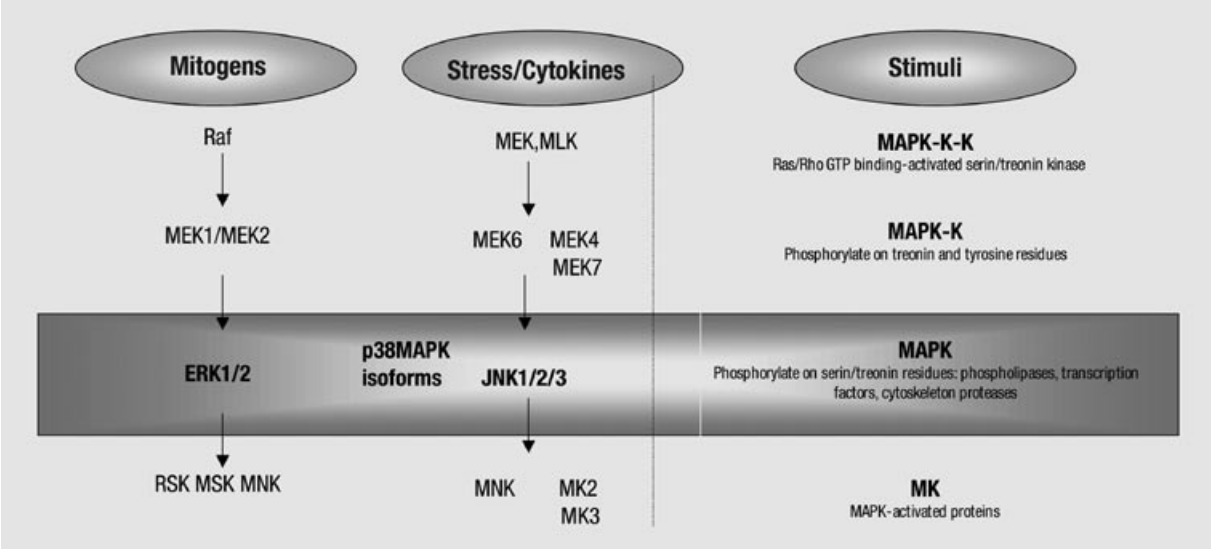

Fig. 5 | *Activation pathways of mitogen-activated protein kinases (MAPKs).*

kinase family and known as MAPKs. MAPKs play an important role in cell proliferation, oncogenesis, differentiation, inflammation and response to stress [91], but several evidence suggest that these kinases is also essential for cell migration [92].

All MAPKs contain tyrosine-X-threonine motifs (where X is any amino acid) in the activation domain and are activated through a kinase cascade in which MAPKKKs (MAPKs kinase kinase) activates MAPKK (MAPKs kinase) which, in turn, activates MAPK by the phosphorylation of threonine and tyrosine residues located in the activation domain *(Figure 5)*. Based on differences in the activation domain, MAPK family can be subdivided into three groups: kinases regulated by extracellular signals (ERK1/2), which display a threonine-glutamine-tyrosine motif, p38 MAPK isoforms which have a threonine-alanine-tyrosine motif, and JNK1/2/3 which present threonine-proline-tyrosin.

Numerous experimental observations have shown that ERK1/2 is directly involved in cell motility [93-95]. Some growth factors and ECM components activate ERK through signalling pathways involving Ras, Raf and MEK1/2. Once activated, ERK phosphorylates different substrates including MLCK, calpain, paxillin and FAK [96-99].

The MLCK phosphorylation determines the focal contact turnover and the formation of cell membrane protrusions at the migrating front [60, 100]. Activated calpain, instead, interacts with the cytoskeleton proteins by promoting focal contact disassembly [95]. Finally, the phosphorylation of FAK and paxillin by ERK may regulate the focal contact dynamics likely by affecting the interaction between FAK and paxillin [97].

Recent studies have shown that p38 MAPK involved in inflammation, apoptosis, cardiomyocyte hypertrophy and cell differentiation, plays a key role in the migration of different cell types, [101-104]. p38 MAPK activity is stimulated by many growth factors, cytokines and chemotactic substances that activate MEK3/6: this protein, in turn, phosphorylates and activates p38 MAPK [105]. p38 MAPK substrates are mainly MAPKAPK2/3 (a protein activated by either MAPK or MK), paxillin and caldesmon which, directly or through the phosphorylation of other proteins, lead to the reorganization of actin and the formation of the "stress fibers" and adhesion structures, thus stimulating the directional migration of cells [106]. Studies on *Drosophila* embryos have shown that JNK is involved in the control of actin cytoskeleton in the formation of filopodia and lamellopodia, and in the movement of cells during neural tube closure [107, 108]. It was also demonstrated that JNK determines the formation of "stress fibers" and their accumulation in the leading front of fibroblasts [109].

RELATIONSHIP BETWEEN DRUG RESISTANCE AND INVASION

In the past studies on drug resistance and invasion generally proceeded along different research paths. Later the interest was focused on the possible relationship between the two phenomena. Currently, many experimental evidence suggest a possible correlation between drug resistance instaurance and acquisition of a highly aggressive phenotype. This relationship has been demonstrated by two observations: (i) drug resistant tumor cells are more invasive and metastatic, when compared with sensitive tumor cells, (ii) in some cases, most metastatic tumors show a greater resistance to chemotherapy [110]. The resistance of tumors to chemotherapy is a

multifactorial and complex phenomenon that often involves different cellular mechanisms [111]. Two types of resistance are known: an "intrinsic resistance" that occurs in cells never exposed to chemotherapy and an "acquired resistance" induced in response to chemotherapeutic treatment [112, 113].

The main cellular mechanisms so far identified, and that play a significant role in the phenomenon of drug resistance can be classified into five groups:

1. drug-target interaction (target resistance);
2. alterations of drug activation, or inactivation, by endogenous biochemical systems present in tumor cell (metabolic resistance);
3. alteration of DNA repair;
4. alteration in the ability of cancer cell to respond to death (resistance to apoptosis);
5. increased drug transport through cell membrane (transport molecule-mediated resistance). After selection for resistance to a single drug, cancer cells may also show cross-resistance to other drugs, structurally and functionally not related [114]. This phenomenon is known as "multidrug resistance" (MDR) and could explain why combined treatments that involve the use of cytotoxic agents with different targets can be not efficacious. MDR acquisition, induced by the treatment with a cytotoxic agent, is generally associated with an increase of transport proteins that results in a reduced intracellular concentration of cytotoxic drug.

The currently known active transporters that play an important role in MDR phenomenon are: members of the P-glycoprotein/TAP (ABCB-P-gp) subfamily *(Figure 6)*, members of the of MDR-associated protein subfamily (ABCC1-MRP1), members of the MXR/BCRP (ABCG) subfamily and the lung resistance related protein (LRP). Except for LRP, all belong to the superfamily of ABC transporters.

If P-gp, MRP and LRP are three important markers of drug resistance, immunohistochemical analyses showed that they are also markers of invasion and metastasis [110, 115]. Moreover, a study on human prostate carcinoma cell lines and its resistant variants with different metastatic capacity [116] gave the following results: cultured, highly metastatic cells express higher levels of bFGF m-RNA, IL-8, MMP-2, MMP-9 and P-gp than poorly metastatic parental cells line. These data suggest a possible correlation between metastasis and drug resistance mediated by P-gp [117]. An *in vivo* study conducted by Bradley *et al.* [118] showed that different stages of tumor progression displayed different levels of P-gp m-RNA expression. Surprisingly, 460 lung metastases were examined and each metastasis was positive for P-gp. Moreover, in an *in vitro* study it was demonstrated that human hepatoma cells rich in P-gp showed increased invasive properties, when compared to cells expressing low levels of P-gp [119]. Bates and colleagues [120] found that a CD44 isoform, normally involved in migration and invasion, coferred chemoresistance to colon carcinoma cells. Also a study of lung carcinoma cells showed that the standard form of CD44 is able to increase the expression of the MRP2 transporter (belonging to the family of "ATP-binding cassette"), resulting in the acquisition of a drug resistance phenotype by NSCLC cell line [121].

The human breast cancer drug resistant cell line MCF-7 ADR which overexpress P-gp shows a different invasive and metastatic potential when compared to the parental line [122, 123] shown that the loss of E-cadherin and the increase of N-cadherin during the acquisition of the resistant drug phenotype correlated with the increase in metastasizing capacity of MCF-7 ADR cells. The variation in expression of cadherin could be due to the control by twist transcription factor. In fact, twist overexpression results in the epithelial-mesenchymal transition (EMT) with the increase in cell motility and invasion. Many studies have shown that the transcriptional activation of the MDR1 transporter is regulated by the MAPK pathway [124, 125], while others have shown that also Snail, a transcription factor that mediates EMT, is regulated by the MAPK pathway as well [126]. Further studies on the involvement of P-gp in the mechanisms of cell migration and invasion have shown that treating MCF-7 ADR cells with the transport substrates of the molecule an increase in the production of CD147, and MMP-2 and MMP-9 is achieved [127]. So the expression and activity of drug resistance genes could simultaneously cross-activate genes that induce tumor metastasis.

Finally, malignant melanoma shows high levels of intrinsic drug resistance associated with a highly in-

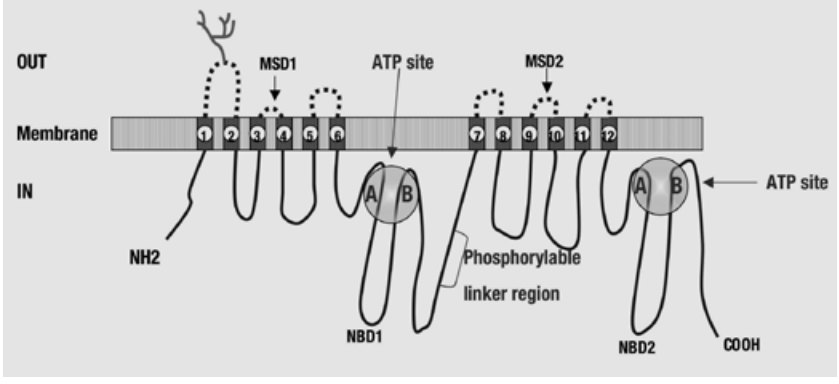

Fig. 6 | *Two-dimensional model of human P-glycoprotein based on the analysis of the amino acid sequence and its functional domains. ATP binding sites, phosphorylation sites, peptide linker, glycosylation sites and 12 transmembrane domains can be identified (Modified from: Di Pietro A, Dayan G, Conseil, G, Steinfels E, Krell T, Trompier D, Baubichon Cortay H, Jault J. P-glycoprotein-mediated resistance to chemotherapy in cancer cells using recombinant cytosolic domain to establish structure-function relationship.* Braz J Med Biol Res *1999;32:925-39). Reproduced with kind permission.*

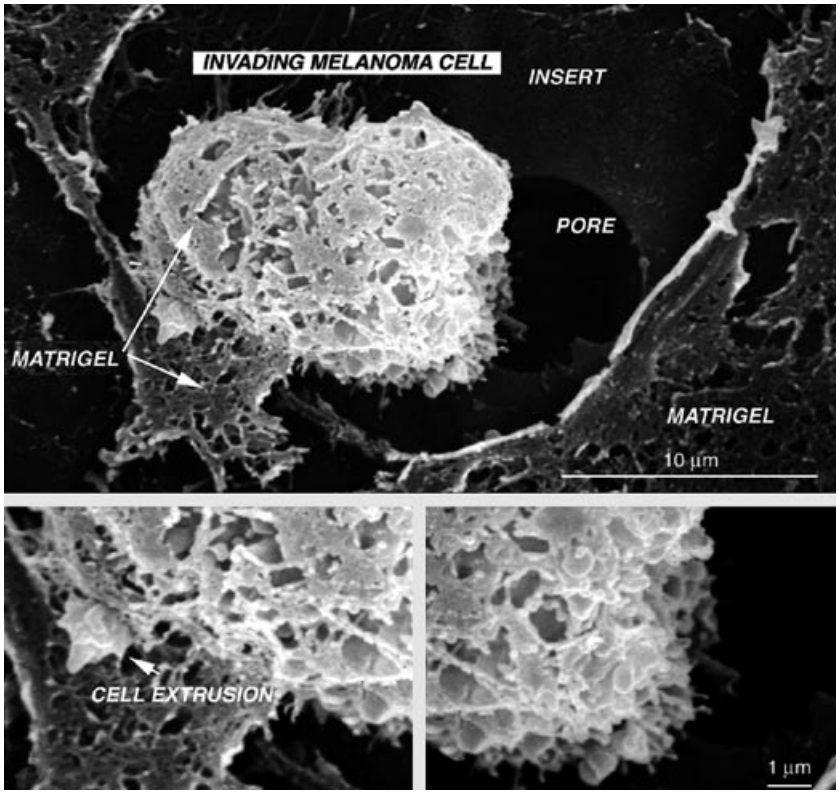

Fig. 7 | *SEM observations of drug-sensitive (M14 WT) cell invasion through Matrigel™. During invasion intense focused proteolysis is visible in sensitive cell samples, indeed the extracellular matrix appears digested around the cell.*

vasive phenotype. CD44, it is the major cell surface receptor to hyaluronan, implicated in cell adhesion, metastasis and tumor progression [128, 129]. When overexpressed in melanoma cells CD44 enhances the experimental metastatic potential and tumorigenicity [128], but its clinical significance in cutaneous melanoma is still unclear.

CD44 proteins assemble intracellular complexes that are important in signal transduction: key players in this regulation are ezrin, radixin and moesin (ERM) proteins. ERM proteins are involved in many physiological functions including regulation of actin cytoskeleton, control of cell shape, adhesion, motility and modulation of signal transduction pathways. In the phosphorylated form ERM proteins anchor CD44 to actin and support cell proliferation [130].

Advances on ezrin involvement in the metastatic phenomenon has been recently reviewed [131]. Novel molecular processes driven by ezrin activation include: phagocytosis, acquisition of resistance to chemotherapeutics and triggering of programmed cell death signals. Federici *et al.*, [132] highlight that ezrin activity is mandatory for both the maintenance of migratory and invasive capacity of tumors and conservation of the ability to feed through other cells. This occurs through the specific molecular association between ezrin and molecules involved in many activities of metastatic cells, such as CD44 and Lamp-1. Particularly, the molecular interaction between ezrin and Lamp-1 was never been described together with its importance in allowing Lamp-1 membrane localization in metastatic cells.

The association of Pgp with actin mediated by ERM family proteins was demonstrated in drug resistant human tumor cells. Such an association appeared to be essential for the maintaining of the MDR phenotype [133]. Successively, a relationship between CD44 and the MDR1 transporter Pgp does exist in carcinoma cell lines [134]. In this study it was stated that the expression of Pgp alone does not increase the migration potential and that it is the interaction of Pgp with CD44 that affects cell migration.

In an our recent study [135], we investigated the role of the drug transporter P-glycoprotein (Pgp) in the invasion potential of drug-sensitive (M14 WT, Pgp-negative) and drug-resistant (M14 ADR, Pgp-positive) human melanoma cells. In particular, co-immunoprecipitation experiments assessed the association of Pgp with the adhesion molecule CD44 in multidrug resistant (MDR) melanoma cells, compared with parental ones. In MDR cells, the two proteins colocalized in the plasma membrane as visualized by confocal microscopy and immunoelectron microscopy on ultrathin cryosections. MDR melanoma cells displayed a more invasive phenotype compared with parental cells, as demonstrated by quantitative transwell chamber invasion assay. The Pgp molecule, after stimulation with specific antibodies, appeared to cooperate with CD44, through the activation of ERK1/2 and p38 MAPK proteins. This activation led to an increase of metalloproteinase (MMP-2, MMP-3, and MMP-9) mRNAs, and proteolytic activities, which are associated with an increased invasive behavior. RNA

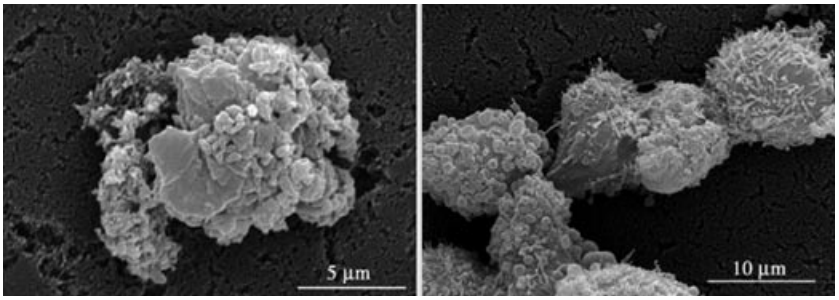

Fig. 8 | *SEM observations of drug-resistant (M14 ADR) cell invasion through Matrigel™. In the lower side of the filter, the invadopodia of resistant cells appeared to infiltrate between the stitches of Matrigel™ in the absence of focused proteolysis.*

interference experiments further demonstrated Pgp involvement in migration and invasion of resistant melanoma cells. A link was identified between MDR transporter Pgp, and MAPK signaling and invasion. Finally, differently from drug-sensitive (Pgp-negative) melanoma cells, which showed an "individual mesenchimal" behaviour *(Figure 7)*, Pgp-overexpressing melanoma cells adopted a 'chain collective' migration strategy reflecting potential high metastatic capacity *(Figure 8)*.

Finally, new perspectives raise from a growing body of literature data which suggest a key role of tumor acidic microenvironment in cancer development, progression, and metastasis [136]. As reviewed in Fais *et al* [136], V-ATPases play a crucial function in determining the acidification of tumor microenvironment and are overexpressed in many types of metastatic cancers and positively correlated to their invasion and metastasis. The promoting effect of V-ATPases on cancer invasion and metastasis mainly relies on their ability to maintain an acidic pH of extracellular microenvironment and very acidic luminal pH. This pathway is in turn related to the activation, secretion, and cellular distribution of many proteases involved in the digestion of ECM. Molecular inhibition of V-ATPases by small interfering RNA *in vivo* as well as a pharmacologic inhibition through proton pump inhibitors (PPIs) led to tumor cytotoxicity and marked inhibition of human tumor growth in xenograft models.

Noteworthy, tumor acidity is also related to tumor cannibalism, a characteristic of malignancy and metastatic behaviour. Through this function metastatic tumors feed off other cells, either dead or alive, including the T lymphocytes that should kill them. Experimental data have shown that cannibalism is increased in acidic culture conditions [137]. A new marker of malignancy with a specific role in tumor cannibalism and in the establishment of metastatic phenotype has been recently proposed [138]. Tumor cannibalism has some similarities to the phagocytic activity of *Dictyostelium discoideum*. Recently, phg1A has been described as a protein that is primarily involved in the phagocytic process of this microorganism. The closest human homologue to phg1A is transmembrane 9 superfamily protein member 4 (TM9SF4). TM9SF4 is highly expressed in human malignant melanoma cells deriving from metastatic lesions, whereas it is undetectable in healthy human tissues and cells. TM9SF4 is predominantly expressed in acidic vesicles of melanoma cells, in which it co-localizes with the early endosome antigens Rab5 and early endosome antigen 1. TM9SF4 silencing induced marked inhibition of cannibal activity, which is consistent with a derangement of intracellular pH gradients, with alkalinization of acidic vesicles and acidification of the cell cytosol.

Received on 16 October 2009.
Accepted on 14 January 2010.

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