THE INDISPENSABLE ROLE OF ADHESIVE STRUCTURES IN ANGIOGENESIS AND METASTASIS

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ABSTRACT:

SUMMARY: Adhesion of cells to the extracellular matrix is essential for a variety of physiological and pathological processes. Different adhesive structures have been described, such as focal adhesions, podosomes and invadopodia. All these structures exert their function through specific adhesion molecules, the integrins, and a variety of signalling molecules. Podosomes have been associated with the process of tumour angiogenesis. Furthermore, invadopodia are characteristic for invasive cancer cells and are linked to tumour invasion and metastasis. Cancer is one of the leading causes of death worldwide and the lack of a proper treatment is a growing problem. Angiogenesis and metastasis are major contributors to mortality in cancer patients. Besides the fact that angiogenesis stimulates tumour growth by increasing the supply of oxygen and nutrients, it also enables tumour cells to metastasize. Since adhesive structures have been associated with these processes, targeting components of adhesive structures could be an addition to the current cancer therapy.

WHAT'S KNOWN: Podosomes and invadopodia are cellular protrusions necessary in physiological and pathological conditions. Among these, angiogenesis and metastasis both contribute to the pathogenesis of cancer.

WHAT'S NEW: This review aims to summarise current knowledge on the role of podosomes and invadopodia in both angiogenesis and metastasis. Furthermore, novel prognostic markers in cancer therapy will be addressed and potentially curative therapies are discussed.

KEYWORDS: Podosomes, invadopodia, metastasis, angiogenesis, cancer treatment

Abbreviations: Arp2/3 complex: Actin related protein subunit 2 and 3 complex; ADAM proteases: a disintegrin and metalloproteinase; alpha-PIX: alpha-PAK-interacting-exchange factor; CDC42: cell division cycle protein 42 homolog; ECM: Extracellular matrix; ECs: Endothelial Cells; FAPα: fibroblast activating protein-α; GFP: Green Fluorescent Protein; HER2: Human epidermal growth factor receptor 2; MMPs: metalloproteinases; MT1-MMP: Membrane Type 1 Matrix Metalloproteinase; N-WASp: Neural WASp; PAK4: p21-associated kinase-4; PDGFRα Platelet Derived Growth Factor Receptor α; RGD: Arg-Gly-Asp; SNARE: soluble N-ethylmaleimide- sensitive factor-activating protein receptor; Src: proto-oncogene tyrosine-protein kinase Src Tks4: Tyrosine kinase substrate 4; Tks5: Tyrosine kinase substrate 5; VEGF: Vascular Endothelial Growth Factor; WASp: Wiskott-Aldrich Syndrome protein

Introduction

Angiogenesis (the formation of new blood vessels) and metastasis are crucial players in mortality in cancer patients [1]. Angiogenesis contributes to the pathogenesis of cancer since it enables metastasis of tumour cells [2]. Cells assemble several structures to adhere to their environment. These adhesive structures have been associated with the processes of angiogenesis and metastasis. The adhesive structures podosomes and invadopodia have both been associated with pathological conditions [3,4]. In this review we describe the role of podosomes and invadopodia in physiological processes and cancer progression to ultimately identify novel prognostic markers and develop targeted therapies.

Podosomes - the cellular feet - and their pathological counterparts: invadopodia

The cellular structures involved in the migration of cells and the degradation of the extracellular matrix (ECM) are called podosomes. Podosomes are ring-like structures that connect with the ECM via integrins which are known to provide a highly stabilised adhesion to the ECM, like feet on a surface, by reorganisation of the actin-cytoskeleton [5]. Podosomes contain a protrusive actin-rich core and are located at the ventral side of a polarized cell, enabling the cell to 'walk' over the ECM. They have been characterised in various cell types such as smooth muscle cells, osteoclasts, macrophages, dendritic cells and endothelial cells [3,5-7]. However, only podosome formation by endothelial cells are associated with angiogenesis promoting cancer progression.

Podosomes fulfil multiple functions in physiological processes. In contrast, invadopodia are protrusion-like structures that are selectively found in invasive tumour cells [4]. Though invadopodia are associated with pathological processes, no complete consensus regarding the similarities and differences of podosomes and invadopodia has been formalized.

In general, two different hypotheses have been described in previous studies as reviewed by Linder et al. [9]. Firstly, it has been suggested that podosomes and invadopodia are different structures and that cell types are not able to express invadopodia and podosomes simultaneously. This is supported by the fact that podosomes have been observed in endothelial cells, smooth muscle cells and monocytic cells, whereas invadopodia are mainly found in highly invasive cancer cells [8,10,11]. Other distinctive features of invadopodia compared to podosomes are the number and size of the adhesive structures, their lifetime and type of ECM degradation. The number of podosomes per cell is higher than invadopodia, namely 20-100 cell-1 compared to 1- 10 cell-1, respectively. Furthermore, invadopodia last up to one hour, whereas the lifetime of podosomes is approximately 2 to 12 minutes resulting in a high turnover rate of podosomes [12,13]. Lastly, while podosomes have a diameter of 0.5 - 1 μM, invadopodia show a diameter of about 8 μM [14]. This difference results from the fact that podosomes induce a relatively broad and superficial degradation of the ECM, whereas invadopodia induce a focused deep degradation enabling tumour invasion (Figure 1) [9,15].

The second theory is based on the argument that podosomes might differentiate into invadopodia, however the complete process has not been experimentally demonstrated yet [16,17]. For podosomes to become functional invadopodia, the size, the lifetime and the total num-

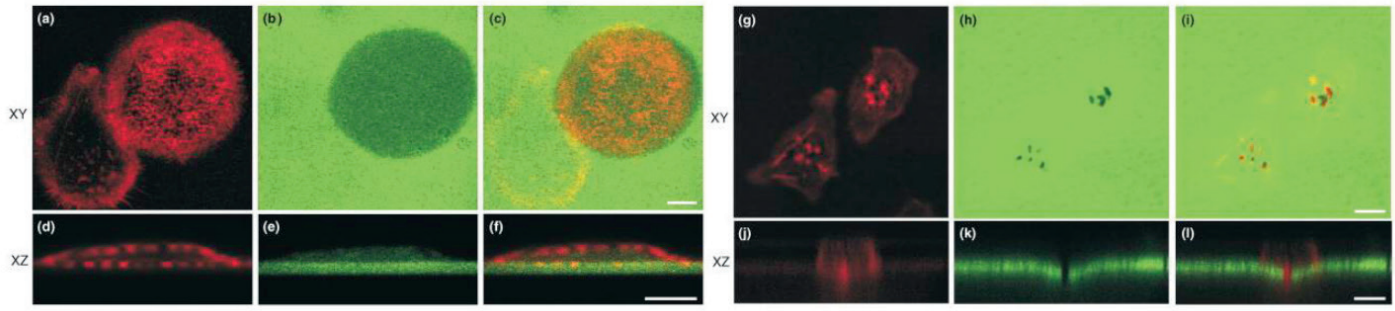

Figure 1 *Matrix degradation by podosomes in macrophages and invadopodia in carcinoma cells. (a-f) Primary human macrophages; (g-l) MTLn3 rat mammary adenocarcinoma cells seeded on Alexa488-labeled fibronectin (green) and stained for F-actin (red); (a,d,g,j) Red channel; (b,e,h,k) Green channel; (c,f,i,l) Merge. Matrix degradation results in a loss of colour, the perpendicular image ('XZ') demonstrates the depth of matrix degradation. Matrix degradation in macrophages is shallow and widespread caused by numerous podosomes. Matrix degradation by sarcoma cells is focalized and deeper caused by a few invadopodia. Figure adapted from Linder et al. [20].*

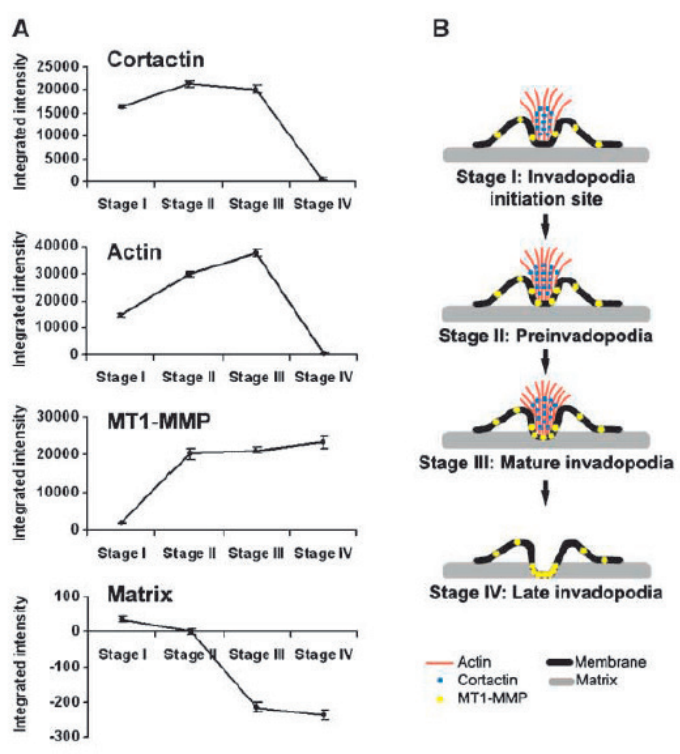

Figure 2 *Model of invadopodia formation and function. (A) Levels of cortactin, actin, and MT1-MMP at invadopodia are given and the degree of matrix degradation is quantified for each invadopodial formation stage. (B) Four stages of invadopodia formation and function are depicted. MT1-MMP: membrane type 1 matrix metalloproteinase. Figure adapted from Artym et al. [42].*

ber of the podosomes should be altered, as reviewed by Linder et al. [9]. Despite the lack of evidence of the actual transition of podosomes into invadopodia, certain characteristics have been described in previously performed studies. As previously mentioned, invadopodia have an increased lifetime compared to podosomes. Experiments using cofilin siRNA in invadopodia showed a decreased lifetime and less matrix degradation that more resembles characteristics of podosomes [18]. These individual processes were described in different experiments. However, it remains unknown whether all processes necessary for transition of podosomes into invadopodia can take place simultaneously. Although there are distinct differences between the two structures, Saltel et al. proposed the term 'invadosome' as an umbrella term for invadopodia and podosomes [19]. The presence of two different theories emphasizes the importance of investigating whether podosomes and invadopodia are different structures or invadopodia are being evolved from podosomes.

Podosome and invadopodia formation

Podosome formation can be initiated through activation of receptor tyrosine kinases by several growth factors. However, integrins are the main receptors responsible for the stimulation of podosome formation [20-22]. Several integrin subunits have been demonstrated to play a vital role in podosome formation [7,23,24].

Podosome formation is dependent on multiple pathways like the Phosphoinositide-3-kinase (PI3K) and Rho-Guanosine triphosphatase (Rho-GTPase) pathway [21,22]. Another downstream key signalling component in the formation of podosomes, is a specific Rho-GTPase called cell division cycle protein 42 homolog (CDC42) [25]. This signalling hub is essential for actin polymerisation and by activation of Wiskott-Aldrich Syndrome protein (WASp) it initiates actin branching [26].

Besides a wide number of structural proteins identified in podosomes, several matrix metalloproteinases (MMPs) have been identified in podosomes of a variety of cell types. Their function is inextricably linked to the ECM degrading capacity of podosomes. However, only the presence, recruitment and function of Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) in podosomes has been thoroughly described. MT1-MMP has a major function in tumour angiogenesis which will be discussed further on.

The process of invadopodia formation depends greatly on similar processes as podosome formation, Artym et al. described this as a four stage process (Figure 2) [27]. Similar to podosome formation, invadopodia formation relies amongst others upon Arp2/3-mediated actin branching at the leading edge of the cell and therefore formation pathways are comparable [28]. Invadopodia formation is being explained in Figure 3.[10].

Podosome involvement in tumour angiogenesis

Angiogenesis plays a major role in metastasis since it facilitates intravasation of primary tumour cells. Angiogenic steps include endothelial cell activation, dissolution of the surrounding basement membrane by MMPs, increased endothelial cell proliferation and migration, tube formation, vessel anastomosis, and pruning to form a vascular network [29]. As mentioned before, endothelial cells possess podosomes that are enriched with MMPs and might therefore be involved in tumour angiogenesis [30].

The physiological processes involved in angiogenesis induce the release of several growth factors including Vascular Endothelial Growth Factor (VEGF) [31]. Recently, Seano et al. identified two distinguishable arrangements of podosomes present in endothelial cells stimulated by VEGF, namely individual podosomes and podosome rosettes at the basal side of the cells, which can be seen in Figure 4A [32]. Rosettes are ring-like structures in which podosomes are clustered together, aggregated by a

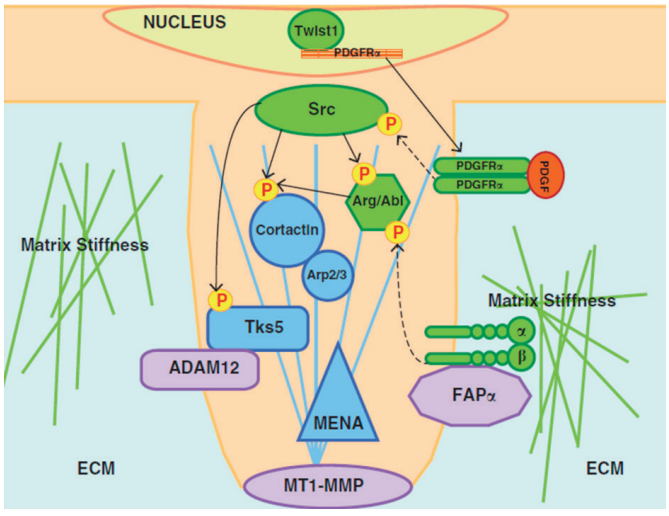

Figure 3 *Main components in invadopodia formation and function. Expression of PDGFRa, which is induced by Twist1, activates Src tyrosine kinase through phosphorylation. The activated Src tyrosine kinase induces invadopodia formation by phosphorylation of Tks5, cortactin and Arg/Abl . Invadopodia assembly triggers the recruitment of various proteases . PDGFRa: platelet derived growth factor receptor-α, Tks: tyrosine kinase substrate, Arg: Abl related gene. Regulatory components are indicated in green; blue indicates structural components of invadopodia which are important for invadopodia assembly (including the actin core); proteases are indicated in purple. Figure adapted from Paz et al. [10].*

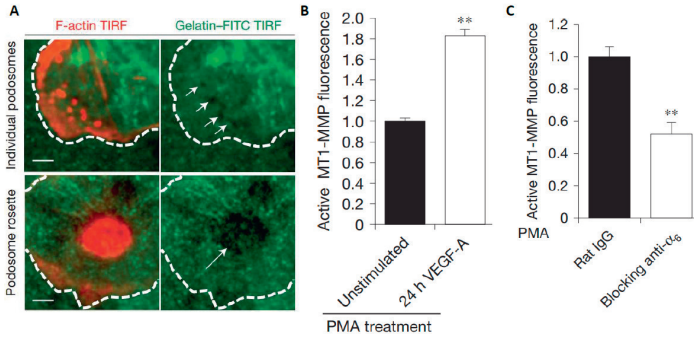

Figure 4 *Podosome and rosette formation stimulated by VEGF in endothelial cells and the inhibition of Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) activity by α6 integrin antibody. (A) To determine proteolytic activity, endothelial cells were stained with phalloidin to visualize F-actin, and seeded on gelatin plates conjugated with FITC. Vascular Endothelial Growth factor-A (VEGF-A) was used to evoke an angiogenic response, and individual podosomes were compared to rosettes in terms of gelatin breakdown. The white dotted lines represent the cell boundaries, and the white arrows represent the decrease in fluorescence and gelatin breakdown. (B) Endothelial cells were treated for 30 minutes with the podosome stimulator phorbol-myristate-acetate (PMA). Angiogenic endothelial cells showed to possess 1.8 fold higher active MT1-MMP levels compared to quiescent endothelial cells, which was statistically significantly ($p<0.01$). (C) Endothelial cells treated with either Rat IgG or anti-α6β1 integrin antibody, followed by PMA treatment for 30 minutes. Addition of anti-α6ß1 integrin antibody significantly reduced MT1-MMP activation its gelatinolytic activity ($p<0.01$). In both B and C, normalized mean +/- SEM is depicted of three individual experiments using 9 x 104 cells. Statistical analysis in these experiments was performed using an unpaired non-parametric Mann-Whitney test. Figures adapted from Seano et al. [34].*

dense network of actin filaments [33]. Besides the induction of podosomes and podosome rosettes in endothelial cells, Seano et al. demonstrated that the activity of MT1-MMP was significantly increased in angiogenic endothelial cells compared to quiescent endothelial cells (Figure 4B) [34]. Furthermore, blocking of α6β1 integrin, a receptor for the basal membrane component laminin, hampered podosome rosette formation and significantly reduced MT1-MMP activity (Figure 4C). Blocking MT1-MMP activity using GM6001 and transfection of cells with siRNA completely abolished the ability for endothelial cells to sprout. In conclusion, blocking the α6β1 integrin may reduce sprouting due to a reduced MT1-MMP activity. This was demonstrated in an in vivo model involving highly angiogenic RipTag2 tumours [34]. Blocking of α6 integrin resulted in a significantly reduced density of endothelial rosettes, followed by a significant decrease in vessel branching. The suggested involvement of rosettes in tumour angiogenesis and the potential ability to block this process might be a target for development of therapeutic strategies to reduce tumour progression and possibly metastasis.

Podosome-targeted therapy

Since cell survival and angiogenesis are crucial factors in tumour progression, integrins that are important for the formation of podosomes might serve as an interesting target for cancer treatment.

For patients that do not respond to current treatment, it might be an option to target upregulated tumour-specific molecules instead. The integrin αvβ3 was found to be abundantly expressed on cancer cells and not on quiescent cells, which makes it an attractive therapeutic target [35]. Since the Arg-Gly-Asp (RGD) tripeptide sequence was proven to be specifically recognized by the αvβ3 integrin this could be utilized for therapeutic purposes [36]. Radiotherapy based on 177Lu-labelled dimeric RGD peptides (177Lu-3PRGD2) is an example of this, which was recently discovered and investigated by Jiyun Shi et al. [36]. Mice that received the targeted radiotherapy 177Lu-3PRGD2 showed significant tumour inhibition compared to saline-treated mice (Figure 6A-B). Mice that received 177Lu-3PRGD2 twice daily exhibited a better tumour inhibition compared to a single dose (Figure 6A). Also treatment with the anti-angiogenic drug "Endostar" showed a significant reduction of tumour growth compared to the saline-treated control group. Mice pre-treated with Endostar for five days before 177Lu-3PRGD2 administration, exhibited a similar degree of tumour inhibition compared to the group receiving both treatments at the same day (Figure 6C-D). Both therapy with 177Lu- 3PRGD2 twice daily as well as combination therapy showed inhibition of tumour growth. Since the combination therapy requires daily injections with Endostar, the two-dose 177Lu- 3PRGD2 therapy is more desirable.

Invadopodia-mediated metastasis

Metastasis can be described as a complex process during which primary tumour cells migrate to distant sites [38]. As reviewed by Fidler et al., this process consists of multiple steps: invasion through the surrounding ECM, intravasation into the bloodstream, transportation via the systemic circulation, and eventually extravasation and formation of new tumours at secondary sites [38]. Invadopodia-mediated ECM degradation is essential during the invasion, intravasation, and extravasation steps of metastasis [39]. ECM degradation by matrix proteases allows the primary tumour cells to migrate.

Invadopodia are able to degrade and remodel the ECM by the recruitment of specific proteases; secreted and membrane-bound MMPs, A Disintegrin And Metalloproteinase (ADAM proteases), and membrane-bound serine proteases. MT1-MMP is a member of the MMPs and plays, as described before, an important role in invadopodia-mediated ECM degradation and remodelling. Perentes et al. demonstrated that down-regulation of MT1-MMP results in a significant decrease in the occurrence of lung metastases which corresponds with reduced cancer cell migration and intravasation [40]. Invadopodia also recruit ADAM proteases, of which ADAM12 has emerged as a prognostic marker for breast cancer and plays an important role in matrix degradation [41]. The last group

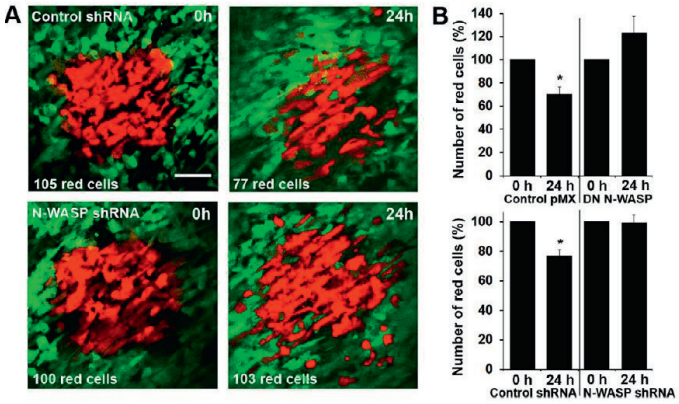

Figure 5 *In vivo intravasation assay of mammary adenocarcinoma MTLn3 cells requires the activity of N- WASP. (A) At 0 hours, the cells with the control shRNA vector at the top, and the cells with the N-WASP shRNA vector at the bottom were converted into a red state by the protein Dendra. At 24 hours, the same cells are shown, and there is more movement of the control shRNA MTLn3 tumor cells into the blood vessels. Scale: 70 µm. (B) Number of red cells remaining around the blood vessel of DN N-WASP and shRNA N-WASP tumours, normalized to the cell number at 0 hours. N-WASP: neural Wiskott–Aldrich syndrome protein. pMX: empty vector. DN: double negative, acts as competitive inhibitor of endogenous N-WASP, as it lacks amino acids for activation of the Arp2/3 complex (top graphs). shRNA: small hairpin RNA, to block N-WASP by silencing N-WASP expression (bottom part). Error bars indicate the SEM (standard error of the mean). * p<0.05 Figure adapted from Gligorijevic et al. [46].*

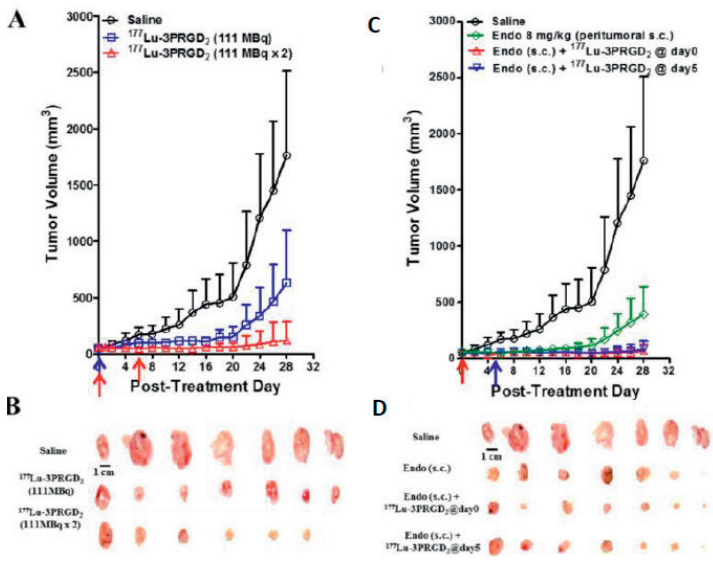

Figure 6 *Radionuclide therapy with 177Lu-3PRGD2 combined with Endostar. (A) Radionuclide therapy of established U87MG tumour in nude mice with saline (as control), 177Lu-3PRGD2 single dose (111 MBq), or 177Lu-3PRGD2 two doses (111 MBq × 2 on day 0 and day 6, respectively). (B) Tumour pictures of the groups depicted in (A) at the end of treatment. (C) Combination therapy of established U87MG tumours in nude mice with saline (as control), Endostar (8 mg/kg, peritumoral subcutaneous injection), Endostar (8 mg/kg, (s.c.) peritumoral subcutaneous injection) + 177Lu-3PRGD2 (111 MBq day 0), or Endostar (8 mg/kg, peritumoral subcutaneous injection) + 177Lu-3PRGD2 (111 MBq day 5). (D) Tumour pictures of the groups depicted in (C) at the end of treatment. The time point of administration of the radioactive compound 177Lu-3PRGD2 (111MBq) was indicated by an arrow, colours indicate corresponding graph. Volume of tumours in each treatment group was measured and expressed as a function of time (means ± SD, n = 7 per group). Figure was adapted from Jiyun Shi et al. [40].*

of proteases are the membrane-bound serine proteases. The serine protease Fibroblast Activating Protein-α (FAPα) was shown to be important in invadopodia-mediated matrix degradation and possibly cooperates with other proteases during this process. The exact role of most serine proteases is not elucidated yet [42]. Degradation of ECM is crucial for tumour cells to metastasize and therefore contributes to tumour progression [43].

To confirm the crucial role of invadopodia in tumour metastasis, it is important to visualize the direct degradation activity of invadopodia. Berginski et al. developed an in vitro model to visualize invadopodia by live cell imaging, although improvements have to be made to avoid false positive results and make this technique implementable [44].

Gligorijevic et al. investigated the importance of invadopodia in the invasion and intravasation steps of metastatic breast cancer by studying the role of Neural-WASp (N-WASp) in vivo [45]. Cancer cells with inhibited N-WASp function showed impaired invadopodia formation and a decreased invasiveness, which suggests that invasion of tumour cells is N-WASp dependent. This invasion appeared to be MMP dependent as well, since introduction of an inhibitor (GM6001) resulted in an impaired invasion of tumour cells in the ECM [45]. In addition to the invasion step, the activity of invadopodia during intravasation into the blood vessel was also investigated by Gligorijevic et al. [45]. Control tumour cells and N-WASp inhibited tumour cells were tracked with a fluorescent protein (Dendra2) to visualize intravasation. After 24 hours, there was no change (or even a minimal increase) in the amount of labeled N-WASp- inhibited tumour cells, which indicates that there was no migration of cells to the bloodstream. This suggests an essential role of N-WASP in the intravasation process (Figure 5). However, in this study, the effect of proliferation was not taken into account. Since proliferation of tumour cells might affect the cell count, the results may be unreliable.

Leong et al. investigated invadopodia and their contribution to in vivo extravasation by real-time 3D time-lapse imaging [46]. They showed that inhibition of cortactin (invadopodia initiation), Tks5 (maturation) and Tks4 (function) resulted in a decreased extravasation. This suggests that disruption of invadopodia via blocking of structural proteins leads to inhibition of metastasis, which provides direct evidence that invadopodia have functional roles during cancer metastasis.

Invadopodia-targeted therapies

Besides targeting components involved in angiogenesis, another target for anti-cancer treatment might be the metastatic process. Invadopodia enhance local invasion and metastasis and are therefore a potential target for the inhibition of cancer metastasis. Several proteins are involved in the regulation of invadopodia formation and function and are therefore interesting targets to inhibit invadopodia formation. DDGFRα activates Src tyrosine kinase to induce invadopodia assembly and thereby promote metastasis. For instance, the selective Src tyrosine kinase inhibitor SU6656 was found to decrease invadopodia formation, as well as the migration and invasion of human breast cancer cells [47]. PDGFRα expression has been identified as a tissue marker for survival in breast cancer patients [48]. The discovery of these important characteristics contributed to the development of several PDGFRα-targeted breast cancer therapies. Sunitinib (Sutent®, Pfizer) is a broad-spectrum tyrosine kinase inhibitor that inhibits PDGFRα amongst other targets. A clinical trial showed a positive effect of Sunitinib treatment in patients with late stage metastatic breast cancer [49]. However, since Sunitinib also targets several cellular components that play a role in invadopodia-independent metastasis, it is difficult to confirm whether the positive effects of Sunitinib treatment are indeed due to the inhibition of invadopodia dependent PDGFRα.

Some of the adverse effects induced by PDGFRα inhibitors might be prevented by the development and use of more specific PDGFRα inhibitors, such as humanized monoclonal antibodies. However, it is possible that highly specific PDGFRα inhibitors do not prevent the metastatic process sufficiently to be clinically beneficial. This implies that it is of crucial importance to obtain the right balance between specificity and efficacy when developing novel therapies targeting PDGFRα.

Another potential target might be MMPs since they have been associated with a poor clinical outcome in breast cancer patients [50-52]. Preclinical trials showed that targeting of several MMPs is effective in reducing invasiveness of cancer cells [53,54], whereas broad spectrum MMP inhibitors have not proven to be successful in clinical trials [55-57]. The low efficiency of MMP inhibitors in clinical trials can be due to the fact that several MMPs exert anti-tumour effects, which are impeded by using MMP inhibitors [58]. For instance, MMP-8 knock-out mice showed an increased incidence of skin tumours, which indicates a paradoxical role for MMP-8 in cancer [59]. Therefore, the strategy of broadly blocking MMPs to prevent metastasis may not be the correct approach, since this may also reduce the anti-tumour effects of certain MMPs. Specific MMP inhibitors might therefore accomplish better results. In addition, it might be interesting to target the system that delivers MMPs to the cellular location of invadopodia. Williams et al. showed that soluble N-ethylmaleimide-sensitive factor-activating protein receptor (SNARE) mediates the trafficking of MT1-MMP. Since trafficking of MT1-MMP is important for ECM degradation during tumour progression, SNARE mediated trafficking might be a potential target for the development of novel therapies [59].

Briefly, specifically targeting integrins or invadopodia-specific pathways might efficiently have a significant anti-tumour effect. Moreover, combining both (target) therapies can lead to even better results in cancer therapy.

Discussion and future perspectives

In recent years, more and more research has focused on the role of podosomes and invadopodia in the processes of metastasis and angiogenesis, which have a considerable contribution to the high mortality seen in cancer patients.

Podosomes are mainly involved in physiological processes. However, they are also known to be involved in tumour angiogenesis. In contrast, invadopodia are specific for invasive cancer cells, but require similar signalling pathways as podosomes. As mentioned before, there is still no consensus reached in literature whether podosomes and invadopodia are similar or distinct structures. Since podosome rosettes are involved in tumour angiogenesis, they might be an interesting therapeutic target to reduce tumour progression and metastasis. It was demonstrated that blocking integrin α6β1 resulted in significantly reduced density of endothelial podosome rosettes, followed by a significant decrease in vessel branching. However, only a decrease in density was observed and not a full elimination of podosome rosettes. This suggests a possible role of escape routes in the formation and/or maturation of podosomes. The presence of compensatory escape routes might also explain the contradictory results that Reynolds et al. observed in β3 null mice, focusing on the involvement of β3 integrin in podosome formation. They showed enhanced angiogenesis, associated with an increased level of VEGF receptor 2 expression, suggesting that angiogenesis takes place in the absence of β3 integrin in these mice [60,61].

Based on the studies of Reynolds et al., it is suggested that αvβ3 has both pro- and anti-angiogenic properties. In the development of the most effective therapy targeting podosomes, complete understanding of the mechanisms and pathways of podosome formation is required. Therefore, contradictory results found in literature need to be elucidated. Overall, αvβ3 may yet turn out to be a good target for anti-angiogenic therapies that target the RGD sequence [37].

The use of novel in vivo detection techniques of the metastatic process may contribute to a better and a more reliable understanding of the different roles of invadopodia during metastasis, and may lead to the discovery of specific targets that might be interesting for the prevention of metastasis. Furthermore, several changes have to be made regarding the research strategy. To start with, there is a large gap between in vitro and in vivo experiments investigating invadopodia-targeted therapies. Only the early steps of metastasis can be investigated using current in vitro models. A tissue-engineered 3D in vitro model, which includes both ECM and blood vessels, might be useful to investigate the role of invadopodia in multiple steps of metastasis. In addition, the tumour environment and the methods of cancer induction in experimental models should resemble the human situation to improve external validity.

Another change that has to be made, is the development of more specific invadopodia-targeted therapies compared to the current non-specific inhibitors. Most therapies, such as PDGFRα- and MMP- targeted therapies, target multiple cellular pathways. This makes it difficult to attribute the potential therapeutic effect to the specific inhibition of invadopodia function. In order to confirm specificity of inhibitors, an invadopodia-specific biomarker should be developed. Furthermore, the implementation of invadopodia inhibitors from preclinical trials into clinical practice is another issue that need to be addressed. The timing of the treatment is crucial for its efficacy, since invadopodia inhibitors only prevent metastasis and do not influence the proliferation and growth of primary tumours. Invadopodia inhibitors can therefore be prescribed when there is (yet) no evidence of metastasis or to prevent metastasis of recurrent tumours.

Since invadopodia share characteristics and underlying formation pathways with podosomes, it is possible that invadopodia targeted therapy also affects podosomes. On top of that, podosomes are in turn involved in multiple physiological processes, therefore side effects may be expected.

Nowadays, a combination treatment of surgery, radiotherapy or chemotherapy combined with anti-angiogenic therapy is performed. Since not all cancer patients respond to conventional therapy, there is a need for other strategies to combat cancer. To improve current practice, treatment according to the 'personalized medicine' principle, for instance by using biomarkers, should be investigated. As it is known for breast cancer, in 20% of the cases there is overexpression of the HER2 gene which can influence the effectiveness of HER2 targeted treatments such as Trastuzumab [62-64].

Conclusion

In conclusion, podosomes are important for angiogenesis, mediating cancer progression. Furthermore, invadopodia also contribute to this progression by promoting metastasis. Invadopodia are specific for invasive cancer cells and invadopodia-specific inhibitors seem to be promising in preventing metastasis of highly invasive cancers. Since podosomes show a lot of similarities regarding signalling pathways with invadapodia, these inhibitors might also affect podosome-mediated angiogenesis. Because of the important role of podosomes - and possibly invadopodia - in several physiological processes, the use of invado-

podia inhibitors might lead to side effects related to those processes. It is important to unravel the exact mechanisms of podosomes and invadopodia initiation and formation to clarify the involvement of these protrusion-like structures in the process of tumour progression. This can eventually contribute to the identification of novel prognostic markers and development targeted therapies. In addition to anti-cancer treatment with cytotoxic drugs, therapies preventing angiogenesis and metastasis might be beneficial in combating metastatic cancer.

References

1. Mehlen P, Puisieux A. Metastasis: a question of life or death. Nat Rev Cancer 2006; 6(6): 449-58.
2. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med 2011; 17(11): 1359-70.
3. Linder S, Nelson D, Weiss M, Aepfelbacher M. Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages. Proc Natl Acad Sci U S A 1999; 96(17): 9648-53.
4. Weaver AM. Invadopodia: specialized cell structures for cancer invasion. Clin Exp Metastasis 2006; 23(2): 97-105.
5. Orlando RA, Cheresh DA. Arginine-glycine-aspartic acid binding leading to molecular stabilization between integrin alpha v beta 3 and its ligand. J Biol Chem 1991; 266(29): 19543-50.
6. Gimona M, Kaverina I, Resch GP, Vignal E, Burgstaller G. Calponin repeats regulate actin filament stability and formation of podosomes in smooth muscle cells. Mol Biol Cell 2003; 14(6): 2482- 91.
7. Zambonin-Zallone A, Teti A, Grano M, et al. Immunocytochemical distribution of extracellular matrix receptors in human osteoclasts: a beta 3 integrin is colocalized with vinculin and talin in the podosomes of osteoclastoma giant cells. Exp Cell Res 1989; 182(2): 645-52.
8. Burns S, Thrasher AJ, Blundell MP, Machesky L, Jones GE. Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. Blood 2001; 98(4): 1142-9.
9. Hoshino D, Branch KM, Weaver AM. Signaling inputs to invadopodia and podosomes. Journal of cell science
10. Paz H, Pathak N, Yang J. Invading one step at a time: the role of invadopodia in tumor metastasis. Oncogene 2014; 33(33): 4193-202.
11. Linder S. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. Trends Cell Biol 2007; 17(3): 107-17.
12. Lener T, Burgstaller G, Crimaldi L, Lach S, Gimona M. Matrix-degrading podosomes in smooth muscle cells. Eur J Cell Biol 2006; 85(3-4): 183-9.
13. Destaing O, Saltel F, Geminard JC, Jurdic P, Bard F. Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. Mol Biol Cell 2003; 14(2): 407-16.
14. Evans JG, Correia I, Krasavina O, Watson N, Matsudaira P. Macrophage podosomes assemble at the leading lamella by growth and fragmentation. J Cell Biol 2003; 161(4): 697-705.
15. Linder S. Invadosomes at a glance. Journal of cell science 2009; 122(Pt 17): 3009-13.
16. Weaver AM. Invadopodia. Curr Biol 2008; 18(9): R362-4.
17. Linder S, Aepfelbacher M. Podosomes: adhesion hot-spots of invasive cells. Trends Cell Biol 2003; 13(7): 376-85.
18. Yamaguchi H, Lorenz M, Kempiak S, et al. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. J Cell Biol 2005; 168(3): 441-52.
19. Gringel A, Walz D, Rosenberger G, et al. PAK4 and alphaPIX determine podosome size and number in macrophages through localized actin regulation. J Cell Physiol 2006; 209(2): 568-79.
20. Saltel F, Daubon T, Juin A, Ganuza IE, Veillat V, Genot E. Invadosomes: intriguing structures with promise. Eur J Cell Biol 2011; 90(2-3): 100-7. 2013; 126(Pt 14): 2979-89.
21. Osiak AE, Zenner G, Linder S. Subconfluent endothelial cells form podosomes downstream of cytokine and RhoGTPase signaling. Exp Cell Res 2005; 307(2): 342-53.
22. Wheeler AP, Smith SD, Ridley AJ. CSF-1 and PI 3-kinase regulate podosome distribution and assembly in macrophages. Cell motility and the cytoskeleton 2006; 63(3): 132-40.
23. Varon C, Tatin F, Moreau V, et al. Transforming growth factor beta induces rosettes of podosomes in primary aortic endothelial cells. Molecular and cellular biology 2006; 26(9): 3582-94.
24. Helfrich MH, Nesbitt SA, Lakkakorpi PT, et al. Beta 1 integrins and osteoclast function: involvement in collagen recognition and bone resorption. Bone 1996; 19(4): 317-28.
25. Schmidt S, Nakchbandi I, Ruppert R, et al. Kindlin-3-mediated signaling from multiple integrin classes is required for osteoclast-mediated bone resorption. J Cell Biol 2011; 192(5): 883-97.
26. Higgs HN, Pollard TD. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu Rev Biochem 2001; 70: 649-76.
27. Wiesner C, El Azzouzi K, Linder S. A specific subset of RabGTPases controls cell surface exposure of MT1-MMP, extracellular matrix degradation and three-dimensional invasion of macrophages. J Cell Sci 2013; 126(Pt 13): 2820-33.
28. Murphy DA, Courtneidge SA. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 2011; 12(7): 413-26.
29. Bowden ET, Barth M, Thomas D, Glazer RI, Mueller SC. An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. Oncogene 1999; 18(31): 4440-9.
30. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 2007; 8(6): 464-78.
31. Seano G, Daubon T, Genot E, Primo L. Podosomes as novel players in endothelial biology. Eur J Cell Biol 2014; 93(10-12): 405-12.
32. Seano G, Chiaverina G, Gagliardi PA, et al. Endothelial podosome rosettes regulate vascular branching in tumour angiogenesis. Nat Cell Biol 2014; 16(10): 931-41, 1-8.
33. Shin Y, Jeon JS, Han S, et al. In vitro 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. Lab Chip 2011; 11(13): 2175-81.
34. Seano G, Chiaverina G, Gagliardi PA, et al. Endothelial podosome rosettes regulate vascular branching in tumour angiogenesis. Nat Cell Biol 2014; 16(10): 931-41, 1-8.
35. Gavazzi I, Nermut MV, Marchisio PC. Ultrastructure and gold-immunolabelling of cell- substratum adhesions (podosomes) in RSV-transformed BHK cells. J Cell Sci 1989; 94 (Pt 1): 85-99.
36. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 1994; 264(5158): 569-71.
37. Kunicki TJ, Annis DS, Felding-Habermann B. Molecular determinants of arg-gly-asp ligand specificity for beta3 integrins. J Biol Chem 1997; 272(7): 4103-7.
38. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech 2011; 4(2): 165-78.
39. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 2003; 3(6): 453-8.
40. Shi J, Fan D, Dong C, et al. Anti-tumor effect of integrin targeted (177)Lu-3PRGD2 and combined therapy with Endostar. Theranostics 2014; 4(3): 256-66.
41. Perentes JY, Kirkpatrick ND, Nagano S, et al. Cancer cell-associated MT1-MMP promotes blood vessel invasion and distant metastasis in triple-negative mammary tumors. Cancer Res 2011; 71(13): 4527-38.
42. Pories SE, Zurakowski D, Roy R, et al. Urinary metalloproteina-

ses: noninvasive biomarkers for breast cancer risk assessment. Cancer Epidemiol Biomarkers Prev 2008; 17(5): 1034-42.
43. Meadows SA, Edosada CY, Mayeda M, et al. Ala657 and conserved active site residues promote fibroblast activation protein endopeptidase activity via distinct mechanisms of transition state stabilization. Biochemistry 2007; 46(15): 4598-605.
44. Chen WT. Proteolytic activity of specialized surface protrusions formed at rosette contact sites of transformed cells. J Exp Zool 1989; 251(2): 167-85.
45. Berginski ME, Creed SJ, Cochran S, Roadcap DW, Bear JE, Gomez SM. Automated analysis of invadopodia dynamics in live cells. PeerJ 2014; 2: e462.
46. Gligorijevic B, Wyckoff J, Yamaguchi H, Wang Y, Roussos ET, Condeelis J. N-WASP-mediated invadopodium formation is involved in intravasation and lung metastasis of mammary tumors. J Cell Sci 2012; 125(Pt 3): 724-34.
47. Leong HS, Robertson AE, Stoletov K, et al. Invadopodia are required for cancer cell extravasation and are a therapeutic target for metastasis. Cell Rep 2014; 8(5): 1558-70.
48. Balzer EM, Whipple RA, Thompson K, et al. c-Src differentially regulates the functions of microtentacles and invadopodia. Oncogene 2010; 29(48): 6402-8.
49. Eckert MA, Lwin TM, Chang AT, et al. Twist1-induced invadopodia formation promotes tumor metastasis. Cancer Cell 2011; 19(3): 372-86.
50. Burstein HJ, Elias AD, Rugo HS, et al. Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. J Clin Oncol 2008; 26(11): 1810-6.
51. Kulic A, Dedic Plavetic N, Vrbanec J, Sirotkovic-Skerlev M. Low serum MMP-1 in breast cancer: a negative prognostic factor? Biomarkers 2012; 17(5): 416-21.
52. Puzovic V, Brcic I, Ranogajec I, Jakic-Razumovic J. Prognostic values of ETS-1, MMP-2 and MMP-9 expression and co-expression in breast cancer patients. Neoplasma 2014; 61(4): 439-46.
53. McGowan PM, Duffy MJ. Matrix metalloproteinase expression and outcome in patients with breast cancer: analysis of a published database. Ann Oncol 2008; 19(9): 1566-72.
54. Kasaoka T, Nishiyama H, Okada M, Nakajima M. Matrix metalloproteinase inhibitor, MMI270 (CGS27023A) inhibited hematogenic metastasis of B16 melanoma cells in both experimental and spontaneous metastasis models. Clin Exp Metastasis 2008; 25(7): 827-34.
55. Mitropoulou TN, Tzanakakis GN, Kletsas D, Kalofonos HP, Karamanos NK. Letrozole as a potent inhibitor of cell proliferation and expression of metalloproteinases (MMP-2 and MMP-9) by human epithelial breast cancer cells. Int J Cancer 2003; 104(2): 155-60.
56. Sparano JA, Bernardo P, Stephenson P, et al. Randomized phase III trial of marimastat versus placebo in patients with metastatic breast cancer who have responding or stable disease after first-line chemotherapy: Eastern Cooperative Oncology Group trial E2196. J Clin Oncol 2004; 22(23): 4683-90.
57. Miller KD, Saphner TJ, Waterhouse DM, et al. A randomized phase II feasibility trial of BMS- 275291 in patients with early stage breast cancer. Clin Cancer Res 2004; 10(6): 1971-5.
58. Fernandez-Garcia B, Eiro N, Marin L, et al. Expression and prognostic significance of fibronectin and matrix metalloproteases in breast cancer metastasis. Histopathology 2014; 64(4): 512-22.
59. Balbin M, Fueyo A, Tester AM, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet 2003; 35(3): 252-7.
60. Williams KC, McNeilly RE, Coppolino MG. SNAP23, Syntaxin4, and vesicle-associated membrane protein 7 (VAMP7) mediate trafficking of membrane type 1-matrix metalloproteinase (MT1- MMP) during invadopodium formation and tumor cell invasion. Mol Biol Cell 2014; 25(13): 2061-70.
61. Reynolds LE, Wyder L, Lively JC, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 2002; 8(1): 27-34.
62. Reynolds AR, Reynolds LE, Nagel TE, et al. Elevated Flk1 (vascular endothelial growth factor receptor 2) signaling mediates enhanced angiogenesis in beta3-integrin-deficient mice. Cancer Res 2004; 64(23): 8643-50.
63. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 2002; 20(3): 719- 26.
64. Gullick WJ, Love SB, Wright C, et al. c-erbB-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 1991; 63(3): 434-8.
65. Artym VV, Zhang Y, Seillier-Moiseiwitsch F, Yamada KM, Mueller SC. Dynamic interactions of
cortactin and membrane type 1 matrix metalloproteinase at invadopodia: defining the stages of invadopodia formation and function. Cancer Res 2006; 66(6): 3034-43.