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CD44, a therapeutic target for metastasising tumours

Véronique Orian-Rousseau *

Karlsruhe Institute of Technology, Institute for Toxicology and Genetics, Hermann von Helmholtzplatz 1, 76344 Eggenstein-Leopoldshafen, Germany

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ABSTRACT

Members of the CD44 family of transmembrane glycoproteins, in particular CD44v6 isoforms, were shown to be metastatic determinants of rat pancreatic tumour cells back in the early 1990s. Furthermore, the expression of several CD44 proteins correlates with aggressive stages of various human cancers. Because of the frequent and homogeneous expression of CD44v6 isoforms in squamous cell carcinoma, antibodies recognising these proteins were used in clinical trials for patients suffering from head and neck squamous cell carcinoma (HNSCC). Although the phase I clinical trials looked promising, the studies were abruptly ended after the death of a patient. Despite the termination of the trials, CD44 certainly remains a valid target for anti-cancer therapy. In this review, alternative strategies targeting CD44 functions are presented. These functions include the binding to hyaluronan (HA), the collaboration with osteopontin and the contribution of CD44 isoforms to receptor tyrosine kinase (RTKs) activation. These new attempts led to the development of peptides that interfere for example with HA binding and that might be used to induce apoptosis in mammary carcinoma or to prevent homing of leukaemia stem cells. Other peptides block RTK activation and thereby inhibit tumour angiogenesis and metastatic spread.

1. The cell adhesion protein family CD44

Together with the selectins, the integrins and the cadherins, CD44 transmembrane glycoproteins form part of the huge family of cell adhesion molecules (CAMs). The CAMs control cell behaviour by mediating contact between cells or between cells and the extracellular matrix and are therefore essential for maintaining tissue integrity. Because of these important functions they are prone to be involved in pathological conditions including tumour progression and metastasis. One of the most striking pieces of evidence that dysregulation of CAMs is involved in tumour progression came from the cadherin field. Indeed, loss of E-cadherin is often associated with an invasive phenotype. However, the first example of CAMs playing a role in the metastatic process came from the CD44 family.

Members of the CD44 family differ in the extracellular domain by insertion of variable regions through alternative splicing (reviewed in Ponta et al.[1]) (Fig. 1). All the isoforms belonging to this family of proteins are encoded by one single gene present on chromosome 11 in humans and chromosome 2 in mice. All isoforms contain a constant region comprising a large ectodomain (270 amino acids), a transmembrane domain (23 amino acids) and a cytoplasmic domain (72 amino acids). These regions are encoded by the first 5 exons and the last exons (16–20) and account for the smallest but ubiquitously expressed isoform, CD44s. Close to the transmembrane region, a variable part encoded by various combinations of exons 6–15 (v1–v10) can be included, giving rise to CD44 variant isoforms (CD44v).

The N-terminal part of the ectodomain enables binding to HA, a huge polymer of repeating disaccharide units of D-glucuronic acid (1-β-3) and N-acetyl D-glucosamine (1-β-4). The

* Tel.: +49 7247826325; fax: +49 7247823354.
E-mail address: veronique.orian-rousseau@kit.edu
0959-8049/$ - see front matter
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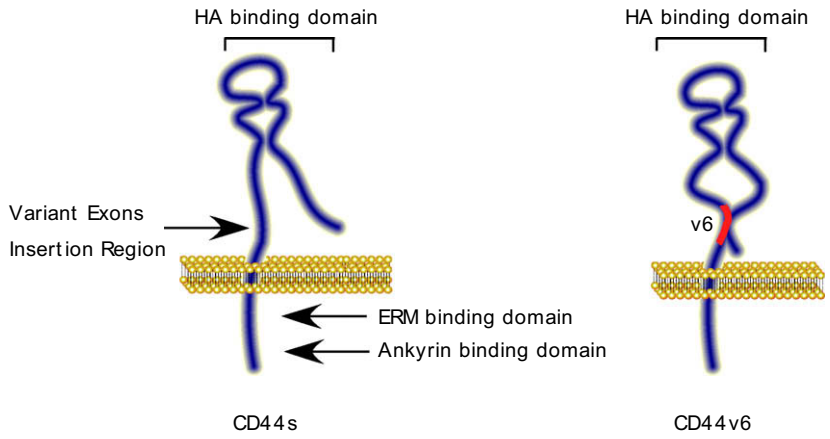

Fig. 1 – Schematic representation of CD44s and one CD44 variant isoform (CD44v6). The important parts of the molecule, namely the HA binding domain, the region of insertion of the variant exons (v1–v10), as well as the binding sites for ankyrin and ERM proteins, are indicated.

HA binding ability is common to all CD44 isoforms. The HA binding domain contains two BX_7B motifs (where B represents an Arg or Lys and X_7 represents any seven non-acidic amino acids) that are also present in other HA binding proteins, such as hyaladherins or the link protein superfamily, the lymphatic vessel endothelial HA receptor-1 (LYVE-1) and Rhamm (Receptor for hyaluronate-mediated motility). The CD44 ectodomain is extensively modified by N- and O-glycosylations, modifications that affect the HA binding ability of CD44 isoforms (reviewed in Ponta et al.[1]). In addition to these modifications, CD44 can be decorated with chondroitin sulphate, or in the case of CD44v3, with heparan sulphate, a modification that makes CD44 an HSPG (heparan-sulphated proteoglycan) and enables it to bind growth factors like HB-EGF, b-FGF[2] or VEGF.[3] The cytoplasmic tail of CD44 binds to ankyrin[4] and to ezrin-radixin-moesin (ERM) proteins,[5] which both provide a link to the cytoskeleton. The binding site for ankyrin is close to a basic stretch of amino acids that anchors ERM proteins to CD44. Phosphorylation of a serine (serine 291) close to the ERM binding site abrogates ERM binding and influences CD44-mediated directional cell motility.[6]

2. Importance of CD44 variants in metastasis

CD44 proteins regulate growth, survival, differentiation and migration and are thereby prone to be involved in tumour progression and metastasis. The first major finding involving CD44 in the metastatic process was the identification of a CD44 variant isoform containing the exons v4–v7 in a highly metastasizing rat pancreatic carcinoma cell line (BSp73ASML). Transfection of this specific variant into the related BSp73AS cells that do not metastasize conferred the metastatic potential to these cells upon injection into syngeneic rats.[7] Furthermore, a CD44 exon v6-specific antibody blocked the metastatic propensity of these cells. Treatment of animals injected with the metastatic cells BSpASv4–v7 with the CD44v6 antibody blocked lymph node and lung metastases.[8]

Since this discovery, a considerable number of studies analysing the expression of CD44 isoforms in human tumours of different origins have been published. The numerous reports are, however, very often contradictory. Overall, it seems that a gain of CD44 isoforms correlates with poor prognosis in several human tumours (for a comprehensive review see Naor et al.[9]). In a limited number of tumours including neuroblastomas and prostate cancer, it is rather the lack of CD44 that correlates with aggressiveness.[10–12] Although a detailed description of the expression pattern of the CD44 isoforms is beyond the scope of this review, a few interesting examples will be mentioned here to highlight the relevance of these isoforms in particular human cancers. In high-grade non-Hodgkin's lymphoma expression of the CD44v6 isoform was detected and correlated with poor prognosis.[13] In colorectal cancer, the expression of CD44s as well as variants is enhanced in adenomas and carcinomas. In addition, expression of CD44s, CD44v3, CD44v6 and CD44v8–v10 correlates with bad prognosis and can be considered a strong prognosticator in patients (reviewed in Wielenga et al.[14]). In cervical cancers, a strong expression of CD44v6 and CD44v7/8 is associated with poor prognosis.[15] In gastric cancer, the expression of CD44v6 and CD44v5 is upregulated and in addition, expression of CD44v5 is preferentially found in poorly differentiated carcinomas and metastatic lymph nodes (reviewed in Hsieh et al.[16]). Finally, in breast cancer, expression of variants containing the exons v3, v6, v7/8 correlates with more aggressive stages of the disease.[17] Whether these CD44 variants can be used as prognostic markers is still a matter of debate.

Isoforms containing the exon v6 seem to be suitable targets for anti-cancer therapy. Indeed, they are causally involved in metastasis of a rat pancreatic carcinoma and in addition, in the human tumours mentioned above, their expression redundantly correlates with tumour progression. Squamous cell carcinoma (SCC), where frequent and homogeneous expression of CD44v6 was detected,[18] was chosen as a suitable model for therapy. Therefore, a high-affinity murine anti-CD44v6 antibody labelled with technetium-99m was first tested in patients suffering from head and neck squamous cell carcinoma (HNSCC). In a phase I clinical study, the distribution of the ^{99m}Tc-labelled CD44v6 monoclonal antibody was specifically detected in the HNSCC tumours.[19–21]

However, as an anti-mouse antibody response was observed in several patients, a humanised version of this antibody (bivatuzumab) was used in the next clinical trials. The humanised IgG1 monoclonal antibody bivatuzumab labelled with rhenium-186 was then used in phase I dose escalation studies in patients suffering from HNSCC. These radiolabelled CD44v6 antibodies showed promising anti-tumour effects with consistent stable disease at higher radioactive dose levels and with only low toxicity.[22,23] Given these promising results, a new strategy was developed using bivatuzumab coupled with a non-radioactive cytotoxic drug[24] instead of radionuclides, raising the possibility of an adjuvant therapy. This compound, named bivatuzumab mertansine, corresponded to the cytotoxic drug mertansine, derived from the antimicrotubule agent maytansine of the benzoansamacrolides family,[25,26] covalently linked to bivatuzumab. In the phase I clinical trial bivatuzumab mertansine was injected weekly for three weeks i.v. into patients with incurable HNSCC. Thirty patients were treated with this compound and three patients showed a partial response. These patients experienced stabilisation of the disease and regression of tumours. As this clinical trial gave a 10% response rate it was considered a success.[27] One patient, however, developed toxic epidermal necrolysis and died. For this reason the development of this drug was terminated.

In addition to HNSCC, patients suffering from early stage breast cancer were also treated with the humanised CD44v6 antibody.[28] In these patients, the ^{186}Re-labelled CD44v6 antibody was well tolerated, but accumulation of the antibody was also detected in non-tumour areas, indicating limitations in the use of this antibody for radioimmunotherapy.

Although the clinical trials for bivatuzumab mertansine were abruptly ended due to skin-related toxicities, it is still clear that it showed some clinical success. Thus, the CD44 isoforms, and, in particular, the CD44v6 isoform, remain crucial targets for tumour therapy.

3. Targeting functions of CD44 relevant to metastasis

3.1. *Influence of HA binding on metastasis*

As mentioned in the introduction, the main property ascribed to CD44 is its ability to bind HA. For this reason, this was the first function of CD44 that was believed to be important for the metastatic process. However, despite intensive research, the data are still contradictory. One attempt to elucidate the role of HA binding to CD44 in tumour progression was made with the pancreatic carcinoma cells BSp73AS transfected with the CD44v4–v7 isoforms that conferred the metastatic potential.[7] Transfection of the same cells with a hyaluronidase expression vector, which resulted in degradation of HA secreted by the tumour cells, did not have any impact on their metastatic propensity.[29]

In contrast, in another cancer cell type, namely the breast cancer cells TA3, the role of HA in tumour invasion was demonstrated.[30] These murine cells were transfected with an expression vector for the CD44 ectodomain (TA3sCD44) and the local release of soluble CD44 by the transfectants inhibited the ability of endogenous cell surface CD44 to bind and internalise HA and to mediate cell invasion into hyaluronan-producing cell monolayers. TA3 cells and their transfectants both adhered to the pulmonary endothelium and penetrated the interstitial stroma when injected i.v. into mice. However, the TA3 cells developed massive pulmonary metastases whereas injection of TA3sCD44 cells induced only a few tumours or no tumours at all. In fact, although the parental cells divided and formed clusters, the TAsCD44 cells underwent apoptosis. These data indicate that CD44 binding to HA supports the metastatic process by inhibiting apoptosis.

In the TA3 cells, binding of HA to CD44 induced CD44 capping. These CD44-HA aggregates were essential for the binding of the metalloprotease MMP9 to the cells and for MMP9 activity. The association of CD44 and MMP9 at the cell surface induced collagen IV degradation and subsequent invasion[31]. Furthermore, MMP9 trapping at the cell surface was necessary for the maturation of the latent form of TGFβ (tumour growth factor β. TGFβ activation triggered, in turn, neovascularisation of the tumour. Overall, MMP9 seems to promote two events: on the one hand, the trapping of MMP9 at the cell membrane by CD44 leads to invasion, and on the other hand, the induction of TGFβ maturation induces angiogenesis.

Thus, inhibition of HA binding to CD44 seems to interfere with events that are crucial for tumour development: inhibition of apoptosis, invasion and angiogenesis.

It seems that the most important event for the trapping of MMP9 by TA3 cells is the clustering of CD44 by HA itself. However, clustering of CD44 can also happen through glycosylation of the variant part of CD44.[32] Therefore, invasion and metastasis might be triggered by these variant isoforms, even in the absence of HA.

Binding of HA to CD44 correlates with invasion and metastasis in many other tumours. As an example, invasion of colon carcinoma cells in matrigel *in vitro* is dependent on CD44 binding to HA and on accumulation of HA in the pericellular region.[33] Indeed, introduction of an antisense construct against two of the three human enzymes responsible for HA synthesis, namely HAS2 and HAS3, inhibited invasion of these otherwise metastatic cells into matrigel. In addition, CD44 antibodies inhibiting the HA-CD44 interaction also blocked invasion of these metastatic cells into matrigel. Along the same lines, introduction of an antisense construct against HAS1 (HAS1-AS) in human bladder cancer cells decreased cell growth and induced apoptosis.[34] Moreover, in xenograft studies the HAS1-AS-expressing tumours grew more slowly than the cells transfected with the control vector and cells overexpressing HAS1. The HAS1-AS-expressing tumours also had much less vascularisation than the recipient bladder tumours.

As mentioned above, the B(X_7)B motif is common to many HA binding proteins. To test whether this motif might be relevant for tumour progression, a 42-amino acid peptide containing three of these domains (designated BH-P) was produced and tested for its anti-tumour activity *in vitro* and *in vivo*.[35] BH-P inhibited tumour growth *in vivo* in chick embryo chorioallantoic membrane (CAM) assays and in nude mice xenograft models using human MDA-435 melanoma cells, B16 melanoma cells or TSU human bladder cancer cells. The anti-tumour activity seemed to be exerted through induction of apoptosis as revealed by activation of critical molecules of the apoptotic pathway, such as caspase-8, caspase-3 or poly (ADR-ribose) polymerase (PARP).

3.2. *Osteopontin and CD44 collaborate to promote cell motility and chemotaxis*

In addition to HA, CD44 has other interacting partners such as collagen, fibronectin, fibrinogen, laminin, chondoitin sulphate, mucosal vascular addressin, serglycin/gp600, the major histocompatibility complex class II invariant chain, L-selectin, E-selectin and osteopontin (OPN) (reviewed in Naor et al.[36]). Amongst all these ligands, the case of OPN seems particularly interesting. Osteopontin is a cytokine that contributes substantially to metastasis in various cancers (reviewed in Weber[37]). The first hints of a possible link between CD44 and OPN came from migration assays where the OPN-induced migration of CD44-transfected fibroblasts in Boyden chambers was blocked by both CD44 and OPN antibodies.[38] OPN could specifically bind to CD44 variants containing the exon v6, but not CD44s in an arginine-glycine-aspartic acid (RGD)-independent manner. This binding of CD44 to OPN was, however, β1 integrin-dependent and led to enhanced cell motility and chemotaxis.[39] Moreover, a 5-kDa fragment of osteopontin (OPN-5kDa) spanning residues 167–210 induced invasion of hepatocellular carcinoma cells (HCC) *in vitro* and this invasion could be inhibited by means of a blocking antibody against CD44.[40] Interestingly, smaller peptides obtained from the OPN-5kDa fragment also blocked the invasion induced by the OPN-5kDa fragment, suggesting a possible therapeutic use against progression of metastatic HCC.

3.3. *Homing of leukaemia cancer stem cells is dependent on CD44*

CD44 is a key regulator of acute myeloid leukaemia stem cells (AML-LSCs). Indeed, homing of these AML-LSCs to their niche and subsequent engraftment is CD44-dependent.[41] An antibody against CD44 blocked leukaemic repopulation in NOD/SCID mice transplanted with human AML cells. The leukaemic burden in these mice was reduced by inhibition of AML-LSC homing and subsequent maintenance in the niche, but also by alteration of their cell fate. This CD44 antibody exclusively targeted the AML cells and not the normal haematopoietic stem cells. This is probably because expression of various CD44 isoforms and in particular CD44v6 are upregulated in the LSCs. Interestingly, CD44v6 has already been shown to be a poor prognostic factor in AML.[42]

Leukaemia stem cells that have an enhanced ability to self-renew are not properly eradicated by current therapies, which exclusively target rapidly cycling progenitors. Therefore, CD44 antibodies, or the more global blocking of CD44 functions, seems to be a promising therapeutic approach to eliminate quiescent AML-LSCs.

The role of CD44 in AML-LSCs might be a result of its collaboration with the cytokine SDF-1 and its receptor CXCR4. Indeed, the homing to and the growth of LSCs in the bone marrow depend on CXCR4 and SDF-1.[43] Interestingly, the homing of haematopoietic stem cells (HSCs) induced by SDF-1 was blocked when the binding of CD44 to HA was inhibited.[44]

CD44 is often cited as a marker of cancer stem cells (CSCs). This is the case for breast cancer, pancreas or colorectal cancers. In all the studies CSCs were screened for CD44 expression with antibodies recognising all CD44 isoforms. Further studies analysing the specific CD44 isoforms expressed on the various CSCs are necessary. Targeting all CD44 proteins, as was shown in the case of AML or of specific CD44 isoforms in CSCs, might therefore interfere with the CSC properties and might thereby be used as a therapeutic strategy in other types of cancers.

3.4. *Involvement of the co-receptor function of CD44 isoforms in the metastatic process*

The mechanism of action of CD44v6-containing isoforms that have been shown to play a prominent role in metastasis[7] has for a long time remained unknown. One property of some CD44 isoforms, namely the ability to bind several growth factors, including HB-EGF, b-FGF[2] or VEGF,[3] and the fact that a CD44v3 heparan-sulphated isoform is necessary for the presentation of FGF to FGFR during limb outgrowth[45] suggested that the role of CD44 in the metastatic process might be linked to its collaboration with receptor tyrosine kinases (RTKs). The possible collaboration between CD44 isoforms and the c-Met RTK was investigated in a first instance and CD44v6-specific antibodies were shown to block c-Met activation in many different cancer cells and primary cells.[46] It turned out that any isoform that contains the v6 exon promotes c-Met activation. The CD44v6 isoform involved in c-Met activation is not heparan-sulphated, indicating that the activation process does not require heparin. HGF, the c-Met ligand, c-Met itself and CD44v6 form a ternary complex that is inhibited in the presence of CD44v6 antibodies.

The role of CD44v6 for c-Met is two-fold: the extracellular domain containing the v6 exon seems to be able to bind to HGF (Matzke and Orian-Rousseau, unpublished data); the cytoplasmic domain in turn recruits ERM proteins and the cytoskeleton in order to promote activation of Ras by its guanidine exchange factor (SOS).[46,47] An ala-scan mutational analysis of the CD44v6 exon enabled the identification of the three amino acids required for activation of c-Met.[48] The important amino acid sequence is EWQ in the rat, GWQ in the mouse and RWH in humans. Most importantly, small peptides spanning this sequence with a minimal size of five amino acids completely abrogated c-Met activation and subsequent downstream signalling.

The CD44 isoform that was identified as a metastatic determinant in the 1990s contained the variant exons v4–v7. Given that a CD44v6 isoform containing exclusively the exon v6 can act as a co-receptor for c-Met, CD44v6 was tested for its ability to induce the metastatic process. This was indeed the case. BSp73AS cells transfected with the CD44v6 isoform (BSp73ASs6 cells) and injected in syngeneic rats metastasised to the lung (Matzke and Orian-Rousseau, unpublished data). The same cells transfected with lentiviral vectors expressing a c-Met shRNA sequence were inhibited in their metastatic process, indicating that c-Met could also confer the metastatic potential to these cells. In order to test whether the co-receptor function of CD44v6 is responsible for the induction of metastasis, rats injected with BSp73ASs6 cells were treated i.v. with the CD44v6 peptides every two to three days. This treatment completely abrogated metastatic spread to the lymph nodes and to the lung (Matzke and Orian-Rousseau, unpublished data).

Interestingly, the co-receptor function of CD44v6 is not restricted to epithelial cells. Indeed, the CD44v6 peptides that inhibit c-Met activation in several epithelial cell types are also able to block activation of c-Met in endothelial cells. Most importantly, the activation of another RTK, namely VEGFR-2, the most prominent RTK in angiogenesis, is also dependent on CD44v6.[49] Consequently, migration of human umbilical vein endothelial cells (HUVECs) induced by VEGF measured in a wound healing assay, as well as the formation of spheroids in matrigel and the formation of tubular networks, were blocked by the CD44v6 peptide. Vascularisation of matrigel/fibrin plugs containing HUVECS mixed with VEGF and injected subcutaneously into nude mice was also blocked by the CD44v6 peptide. These peptides also completely abrogated both angiogenesis of pancreatic tumours obtained after injection of the pancreatic carcinoma cells l3.6 pl orthotopically in the pancreas of nude mice[49] and metastasis to the liver (Matzke and Orian-Rousseau, unpublished data).

Taken together, these data indicate that the CD44v6 peptides are able to block metastasis of pancreatic carcinoma cells injected into syngeneic rats as well as metastasis of human pancreatic cells orthotopically injected into the pancreas of nude mice. Therefore, they represent a promising therapy against cancer (Fig. 2), in particular pancreas cancer, where the survival rate is extremely poor. The efficiency of the CD44v6 peptides is currently being tested in orthotopic and xenograft models using breast or oesophagus cancer cells. In addition, modified peptides, peptidomimetics and acid-degradable polymer nanocarriers coupled to CD44v6 peptides are being produced in order to improve their efficiency.

4. Outlook

There is ample evidence that CD44 isoforms and specifically the CD44v6 isoforms are involved in the metastatic process. Several studies show that the function of CD44 as an HA binding protein is crucial for tumour progression, although this seems not to be a common feature for all tumours. Furthermore, the co-receptor function of CD44v6 for c-Met and also for VEGFR-2 might be crucial for the establishment of primary tumours as well as for metastasis, at least in pancreatic carcinoma. For these reasons, CD44 proteins, and, in particular, the CD44v6 isoform, are interesting targets for tumour therapy. In addition, the phase I clinical trial performed with CD44v6 antibodies that were either radiolabelled or covalently linked to a toxin in patients affected by HNSCC gave promising results. Unfortunately, in the cohort of patients treated with the toxin-conjugated antibodies, one patient died, most probably from side effects, and the trial was terminated. A possible therapy using the CD44v6 peptides that block the function of at least two different RTKs and interfere with tumour angiogenesis and metastatic spreading might offer a new therapeutic route with fewer side effects. Furthermore, the fact that CD44 seems to be a reliable marker of several types of cancer stem cells increases the interest in these molecules and in their use in tumour therapy.

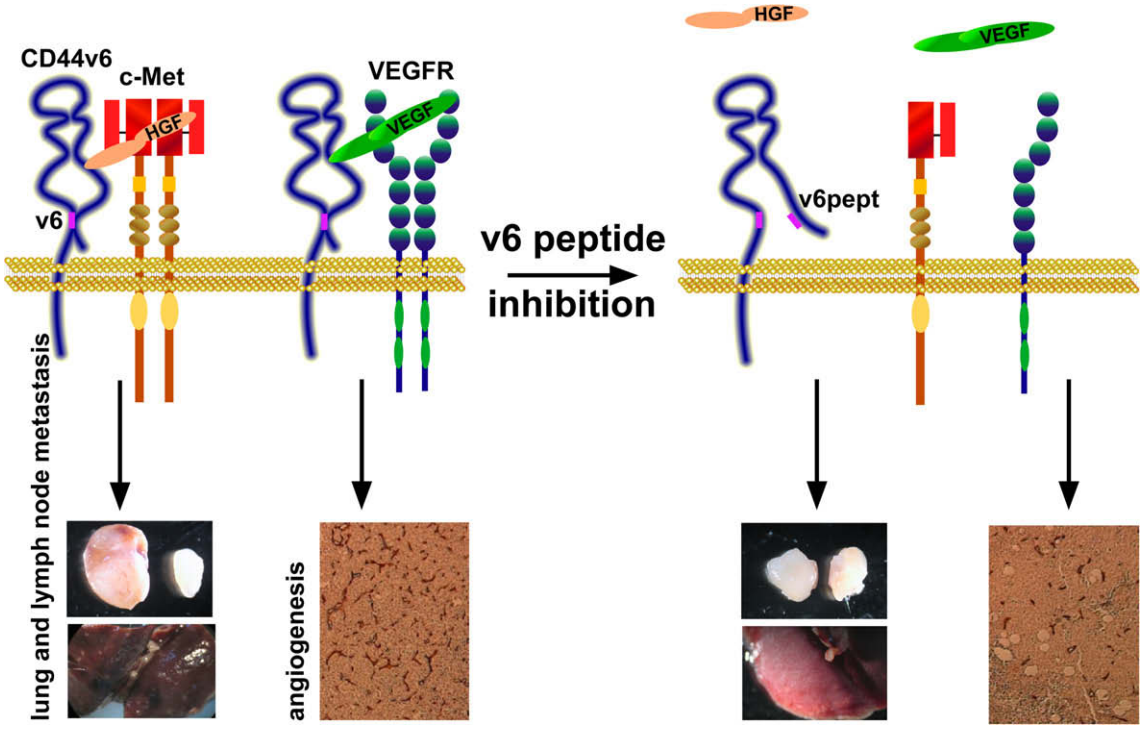

Fig. 2 – The CD44v6 peptides: promising tools against metastatic spread. The co-receptor function of CD44v6 for c-Met and VEGFR-2 promotes metastatic spread of the BSp73ASs6 rat pancreatic carcinoma cells to the auxiliary lymph nodes and to the lung and neovascularisation of MDA-MB231 human breast tumours. Small peptides originating from the CD44v6 exon with a minimal size of five amino acids change CD44v6 conformation and prevent binding of the RTK ligands, thereby blocking metastasis and angiogenesis.

Conflict of interest statement

None declared.

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