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Oral-examination:

Isoform-specific Loss of CD44 Interferes with Different Aspects of the Metastatic Process

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Whenever you fall,

pick something up.

Oswald Theodore Avery

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Summary

The cell-cell and cell-matrix adhesion molecule CD44 and its numerous splice variants are involved in a multitude of physiological and pathological processes, including tumour progression. Especially variant CD44 has been implicated in metastasis formation.

For long term *in vivo* experiments on metastasis formation, a plasmid based RNAi technique was applied to generate stable splice variant 'v7'-specific CD44 knock-down clones of a highly metastatic rat pancreatic adenocarcinoma cell line (BSp73ASML). The resulting phenotype was characterized with an emphasis on interactions of CD44v with the tumour surrounding during the course of metastasis formation. Loss of CD44v is accompanied *in vivo* by a marked reduction in metastatic growth in the lymph nodes and particularly in the lung, which could be reverted by restoring CD44v expression in the knock-down cells. The impaired metastatic growth was not due to a lower proliferative activity or a reduced anchorage-independence of these cells *in vitro*. Instead, they display several defects, which can be attributed to perturbed interactions of CD44v with the microenvironment. Compared to ASMLwt cells CD44vk.d. cells do not form cell aggregates in stromal surroundings, such as lymph nodes and the lung, due to lost cell-cell adhesion, mediated by interactions of CD44v and hyaluronic acid (HA). Furthermore, CD44vk.d. cells exhibit an impaired matrix production, as CD44v is most likely involved in the assembly of matrix components, containing HA, collagen and laminin. The matrix supports rapid adhesion of ASML cells through β1 integrin and in addition contributes to survival. Finally, the loss of CD44v is accompanied by a marked decrease in apoptosis resistance. Impaired PI3K-Akt survival signalling, activated by CD44v was identified as the cause of this defect.

In conclusion, CD44v contributes to the metastatic phenotype of ASML cells as a multifunctional player interacting with the surrounding in several ways. First, as cell-cell adhesion molecule by mediating cell aggregation, second, as cell-matrix adhesion molecule by organizing matrix generation and last, as signalling molecule supporting survival. This highlights the role of variant CD44 in the metastatic spread of tumour cells through complex interactions with the tumour microenvironment and underlines the important role of a highly regulated interplay between tumour cells and their surrounding for metastasis formation.

Zusammenfassung

Das Zell-Zell und Zell-Matrix Adhäsionsmolekül CD44, sowie seine zahlreichen Spleißvarianten sind an einer Vielzahl physiologischer und pathologischer Prozesse beteiligt, zu denen auch die Tumorprogression zählt. Besonders variante CD44 Formen wurden mit Metastasierung in Verbindung gebracht.

Um langwierige *in vivo* Experimente zur Untersuchung von Metastasenausbildung zu ermöglichen, wurden stabile CD44-'knock-down'-Klone einer stark metastasierenden Pankreas-Adenokarzinomlinie der Ratte (BSp73ASML) generiert. Über plasmidbasierte 'RNA interference' (RNAi) wurde die CD44-Expression spleißvarianten-'v7'-spezifisch reguliert. Der resultierende Phänotyp wurde besonders im Hinblick auf Interaktionen zwischen CD44v und der Mikroumgebung im Verlauf der Metastasierung charakterisiert. *In vivo* führt der Verlust von CD44v zu deutlich reduzierter Metastasenbildung in den Lymphknoten und besonders in der Lunge, und dieser Effekt war durch wiederhergestellte CD44v-Expression in den 'knock-down'-Zellen wieder umkehrbar. *In vitro* zeigen die CD44v-k.d.-Zellen unveränderte Proliferationsaktivität und das gleiche verankerungsunabhängige Wachstumsvermögen wie der Wiltyp. Demgegenüber weisen sie mehrere Defekte auf, die auf Interaktionsverlust von CD44v mit der Mikroumgebung beruhen. In stromaler Umgebung, wie in den Lymphknoten und der Lunge, bilden CD44v-k.d.-Zellen im Gegensatz zu ASMLwt-Zellen keine Zellaggregate aus, was auf den Verlust von CD44 und Hyaluronsäure vermittelten Zell-Zell-Kontakten zurückgeführt werden konnte. Zusätzlich ist die Matrixproduktion dieser Zellen beeinträchtigt, da CD44v höchst wahrscheinlich eine Rolle bei der 'Matrixmontage' zukommt. Als Bestandteile der Matrix konnten Hyaluronsäure, sowie Laminin und Kollagen identifiziert werden. Die Matrix ermöglicht ASML-Zellen eine rasche über β1-Integrin vermittelte Adhäsion und trägt darüberhinaus zum Überleben der Zellen bei. Schließlich geht der Verlust von CD44v mit einer deutlichen Abnahme der Apoptoseresistenz einher. Als Ursache für diesen Defekt konnte eine beeinträchtigte PI3K-Akt-Signaltransduktion identifiziert werden, die durch CD44v aktiviert wird.

Zusammenfassend konnte gezeigt werden, daß variantes CD44 maßgeblich zum metastasierenden Phänotyp von ASML Zellen als multifunktionales Molekül beiträgt, indem es mit der Mikroumgebung auf verschiedene Art und Weise interagiert. Zunächst als Zell-Zell Adhäsionsmolekül, das Zellaggregation vermittelt, dann als Zell-Matrix

Adhäsionsmolekül, das die Bildung der Matrix organisiert, und schließlich als Signaltransduktionsmolekül, das zum Überleben der Zelle beiträgt. Diese Ergebnisse heben die Rolle von variantem CD44 für die Metastasenbildung von Krebszellen durch komplexe Interaktionen mit der Tumor-Mikroumgebung hervor und unterstreichen die entscheidende Bedeutung von komplex regulierten Wechselwirkungen zwischen Tumorzellen und ihrer direkten Umgebung für die Metastasierung.

1. Introduction

30 years of intensive research in the field of tumour biology produced a huge body of knowledge and the basic mechanisms underlying the onset and progression of cancer have been identified. Nonetheless, after cardiovascular diseases cancer is still the 2nd leading cause of death in the western world (WHO, 2003).

Cancer arises from a single cell that underwent genomic alterations leading to gain-of-function of so called oncogenes or loss-of-function of tumour suppressor genes enabling uncontrolled growth and evading the bodies defence system to eliminate cells with dysfunctions. Our understanding of the mechanisms underlying early tumour progression is steadily growing, concomitantly with remarkable advances in the diagnosis and treatment of early tumours. However, there is still little understanding of the late steps in tumour progression leading to metastasis formation, which causes 90% of human cancer deaths (Storm, 1996). Although there is a growing number of genes being identified to take part, the underlying mechanisms that enable cancer cells to disseminate from the primary tumour mass and settle at distant sites in the body to form metastases is still poorly understood and deeper insights are needed for future therapeutic strategies to treat metastatic cancers.

1.1 Cancer evolves as a multistep process

During tumourigenesis the transformation of a normal cell into a malignant cancer follows a multistep process, which can be understood as an evolutionary event following the Darwinian concept. In order to develop into a life threatening invasive tumour a cell has to acquire certain characteristics, reflecting genetic alterations, which confer a growth advantage and drive the progressive transformation of the cell (Foulds, 1954; Nowell, 1976). Hanahan and Weinberg proposed six essential capabilities required for metastatic cancer formation (Hanahan and Weinberg, 2000), namely self sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis.

Self sufficiency in growth signals

Normal cells need growth stimuli in order to proliferate. These signals can be diffusible growth factors, extracellular matrix components or cell-cell stimulations. Usually secreted by other cell types (heterotypic signalling) these signals are sensed mainly by transmembrane receptors (binding to diffusible growth factors) and integrins (binding to components of the ECM), which translate the outside stimulus into an inside signal. Many cancer cell lines are independent on such exogenous growth stimulation because the activity of oncogenes mimics these growth signals by modulating the underlying stimulatory machinery at different levels. Tumour cells can either secrete their own growth factors (autocrine stimulation) or modify the corresponding signals within the cell, by modulating the receptors itself or the downstream signalling circuits. Alteration of growth factor receptor signalling can be achieved at the expression level leading to hyper-responsiveness to a given extracellular signal or by modulation of the signalling ability of the receptor, like expressing constitutively active versions of the receptor. A prominent example for this is the truncated version of the EGF-receptor (Fedi, 1997). Alternatively, the underlying growth signalling circuits itself can be modulated. The Ras-Raf-MAP kinase pathway for example is altered in 25% of human tumours leading to mitogenic signals without ongoing upstream stimulation (Medema and Bos, 1993). Cells are also dependent on growth stimuli from the underlying ECM. Many cell-matrix interactions are regulated by integrins, which are heterodimeric cell surface adhesion molecules composed of one α and one β subunit. To date, 18 different α and 8 different β subunits have been identified, which form at least 24 heterodimers with different characteristics (Hynes, 2002; Shimaoka and Springer, 2003). Cancer cells can change their integrin repertoire by varying the combination of α and β subunits, for instance to favour expression to integrins eliciting progrowth signals at the expense of integrins with antiproliferative effects. In addition to this enhanced autonomy from their surrounding, tumour cells are able to modulate the behaviour of their neighbourhood for their own benefit. For example cancer cells can induce excess release of growth factors by neighbouring cells (Skobe and Fusenig, 1998) or stimulate inflammatory cells that should rather eliminate tumour cells, to promote their growth instead (Cordon-Cardo and Prives, 1999; Coussens et al., 1999; Hudson et al., 1999).

Insensitivity to antigrowth signals

Just as normal cells display dependence on progrowth signals they are sensitive to antigrowth signals. This ensures tissue homeostasis and cellular quiescence. Antiproliferative signals can, like their positively acting counterparts, be soluble growth inhibitors and immobilized inhibitors present in the ECM or on neighbouring cells that are sensed by transmembrane receptors. Such inhibitory stimuli may lead to cell cycle arrest or induce a permanent mitotic stop by driving the differentiation of the cell. Most antiproliferative signals are relayed via the 'retinoblastoma protein' (*pRb*) pathway. *PRb* is a classical tumour suppressor gene, which controls progression from G1 to S phase of the cell cycle (Weinberg, 1995). Cancer cells often fail to respond properly to antigrowth signals due to disruption of this pathway. Inactivation of the pRb protein leads to cell cycle arrest, either by mutation of the gene itself or by interfering with 'transforming growth factor β' (TGFβ) signalling, which normally blocks phosphorylation and thereby inactivation of pRb (Hannon and Beach, 1994; Datto et al., 1997). Blocking TGFβ signalling is achieved by cancer cells through down-regulation of the corresponding receptor, by expression of dysfunctional receptors (Fynan and Reiss, 1993; Markowitz et al., 1995) or by modulating downstream signalling events (Schutte et al., 1996; Chin et al., 1998). Integrins can elicit antigrowth signals as well, and their expression pattern exerting proliferative or antiproliferative effects can be modulated as described above. Another way to avoid growth arrest is to circumvent differentiation. For example, antagonizing c-Myc function can lead to cellular differentiation (Dang et al., 1999) and recently a role for pRb in this context was shown as well (Goodrich, 2006).

Evading apoptosis

The third aspect limiting the expansion of a cell population is controlled by cross talk between the cell and the environment, deciding if a cell should live or die. Programmed cell death or apoptosis is the major event regulating this subject and it is evident that resistance to apoptosis is a key feature of cancer cells and for metastasis formation. Programmed cell death is a mechanism common in virtually all cell types and follows the same routes once it is induced by a variety of different triggers. Cell membranes are disrupted, the cytoskeleton and chromosomes are degraded and the cell usually dies within 24 hours (Wyllie et al., 1980). Apoptosis can be induced via two distinct routes, depending on the source of the apoptotic stimulus. The extrinsic or death receptor pathway is induced by surface receptors like Fas and 'tumour necrosis factor receptor' (TNFR) that bind their corresponding ligands and induce a

cellular signalling cascade eventually leading to apoptosis. On the other hand there are several intracellular sensors detecting abnormalities within the cell, such as DNA damage, survival factor insufficiency or other stress signals like hypoxia (Evan and Littlewood, 1998). Such triggers induce the intrinsic or mitochondrial pathway, usually by activating pro- or inhibiting antiapoptotic members of the Bcl-2 protein family. These signals lead to breakdown of the mitochondrial membrane potential, release of cytochrome c and finally to apoptosis (Green and Reed, 1998). The *p53* tumour suppressor gene for example, which is inactivated in more than 50% of human cancers, exerts its apoptotic effects via the intrinsic pathway. In response to DNA damage p53 upregulates the proapoptotic Bcl-2 protein Bax, which leads to cytochrome c release from the mitochondria (Harris, 1996). Both the external and intrinsic pathway finally converge on the ultimate effectors of apoptosis, intracellular proteases called caspases (Thornberry and Lazebnik, 1998). Two initiator caspases 8 and 9 are activated either by death receptor signalling or by cytochrome c release, which then in turn activate several effector caspases, executing downstream steps of the apoptotic program and eventually leading to degradation of cellular structures, organelles and the genomic material (Fig. 1).

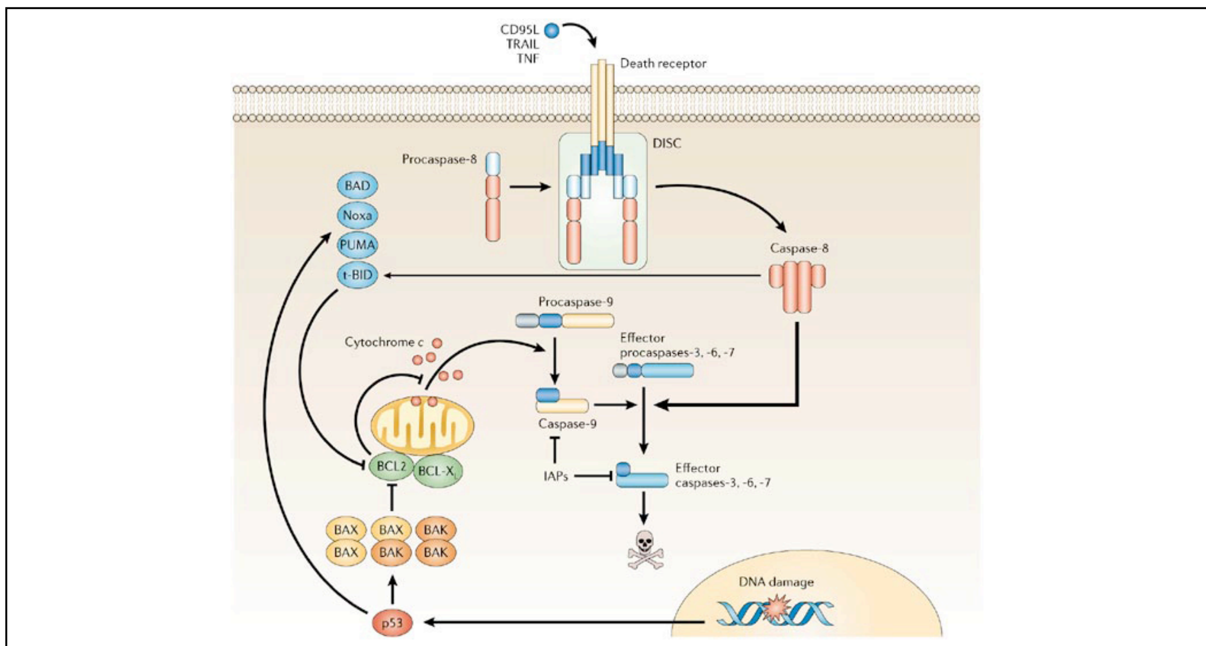

Fig.1: The two main death pathways of apoptosis
Death receptors on the surface of the cell bind their cognate ligands, leading to engagement of the 'death inducing signalling complex' (DISC) and to activation of caspase 8. Internal stimuli, such as genotoxic stress induce apoptotic molecules, finally leading to inhibition of antiapoptotic molecules of the Bcl-2 family, subsequent cytochrome c release from the mitochondria and caspase 9 activation. Ultimately, both routes lead to activation of effector caspases and cell death. IAP inhibitor of apoptosis proteins. *Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Mehlen and Puisieux., 2006).*

Cancer cells escape from apoptosis either by deregulating the expression of death receptors on their surface or by survival signals, counteracting apoptotic stimuli through induction of antiapoptotic molecules. One of the best-studied pathways in this regard is the PI3kinase-Akt pathway. PI3K-Akt can be influenced by external stimuli, for example IGF or IL-3 (Evan and Littlewood, 1998), or by intracellular signals, e.g. the *ras* oncogene pathway (Downward, 1998) or the loss of the tumour suppressor pTEN, which normally attenuates Akt survival signals (Cantley and Neel, 1999). Likewise, cell-cell and cell-matrix interactions are necessary for cell survival. Apoptosis due to loss of adhesion to the substratum is called anoikis and provides another important mechanism to control cellular behaviour.

In summary, apoptosis is regulated by an extremely complex machinery of pro- and antiapoptotic signals, which are balanced in a healthy cell and shifted out of this equilibrium in cancer cells.

Limitless replicative potential

In addition to signals regulating the growth properties of a cell in response to its environment, the life span of a normal cell underlies an intrinsic program limiting its replicative potential. Non-transformed cells in culture have the capacity to divide only about 60-70 times. With increasing cell divisions they undergo a process termed senescence and stop growing. This process can be circumvented for example by blockage of the *pRb* and the *p53* tumour suppressor genes, enabling the cell to further multiply until a second state called crisis, is reached. This state is characterized by massive cell death and increased karyotypic rearrangements, like chromosome fusions, and eventually enables a cell to replicate without limit (Wright et al., 1989). Most isolated tumour cells demonstrate this immortalization in culture, arguing for immortalization as a prerequisite for cancer to evolve. However, theoretically 60-70 doublings are more than enough for a cell to expand to a life-threatening tumour mass. On the other hand, the apoptotic rate within tumours is high and the actual cell number of a tumour greatly under represents the cell generations required to produce it. As a result of this, limitless replicative potential would be a prerequisite for cancerous transformation.

The molecular mechanism underlying senescence is based on the replication of the genomic material. During each cell cycle the whole genome is replicated but the ends of the chromosomes, called telomers are shortened through each round of replication, due to the inability of the appropriate polymerase to replicate the chromosome completely. Telomers do

not carry any genomic information, but protect the ends of the chromosomal DNA. They prevent end to end fusions and karyotypic misarrangements, which are associated with crisis and result in the death of the cell (Counter et al., 1992). Telomer stability is observed in nearly all types of cancer and cancer cells maintain the integrity of their telomers by over-expressing an enzyme called telomerase, which elongates the telomeric DNA and compensates for the shortening during replication (Bryan and Cech, 1999). To a lesser extent, telomer maintenance in cancer cells can also be achieved by recombination-based interchromosomal exchanges of sequences termed 'alternative lengthening of telomers' (ALT) (Bryan et al., 1995).

Sustained angiogenesis

Every growing tissue is dependent on supply with nutrition and oxygen, which is guaranteed in healthy tissue by a dense network of blood vessels. Tumours also depend on the vasculature to be able to grow beyond a certain size. During tissue development the formation of blood vessels from pre-existing vessels (angiogenesis) and the *de novo* formation of new blood vessels (neovascularization) is regulated by complex interactions between the tissue and the endothelial cells of the vasculature. Positive and negative regulators of angiogenesis are tightly balanced and transiently regulated in order to induce blood vessel outgrowth. Mainly soluble factors, like 'vascular endothelial growth factors' (VEGFs) and 'fibroblast growth factors' (FGFs), which attract endothelial cells expressing the cognate surface receptors (Veikkola and Alitalo, 1999) or inhibitors of angiogenesis like thrombospondin (Bull et al., 1994) regulate the process of neovascularization. In order to grow out into a macroscopic tumour, cancer cells have to interfere with this programme by shifting the balance to the angiogenic inducers and counteracting the inhibitors (Hanahan and Folkman, 1996). Indeed many tumours reveal enhanced expression of VEGFs and FGFs and impaired expression of thrombospondin (Singh et al., 1995; Volpert et al., 1997). Activators or inhibitors of angiogenesis can also be stored in the ECM and released by proteases, that are expressed by tumour cells (Whitelock et al., 1996). The importance of sustained angiogenesis for tumour growth has been shown in several studies (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997) and is a target for therapeutic intervention.

Tissue invasion and metastasis

90% of human cancer deaths are due to the formation of secondary tumours (Sporn, 1996) arising from cells, that moved out of the primary tumour mass and settled at distant sites in the body. The metastatic spread of tumour cells can be understood as a multistep process itself. In order to settle at a secondary site and to form a new tumour, a cancer cell has to acquire the ability to break down cell-cell and cell-matrix connections, invade adjacent tissues by overcoming tissue borders and acquire a migratory phenotype. Then, metastatic cells have to enter the blood circulation or the lymphatic system to be transported to distant sites, where they have to exit the circulation again in order to form secondary tumours (Fig.2).

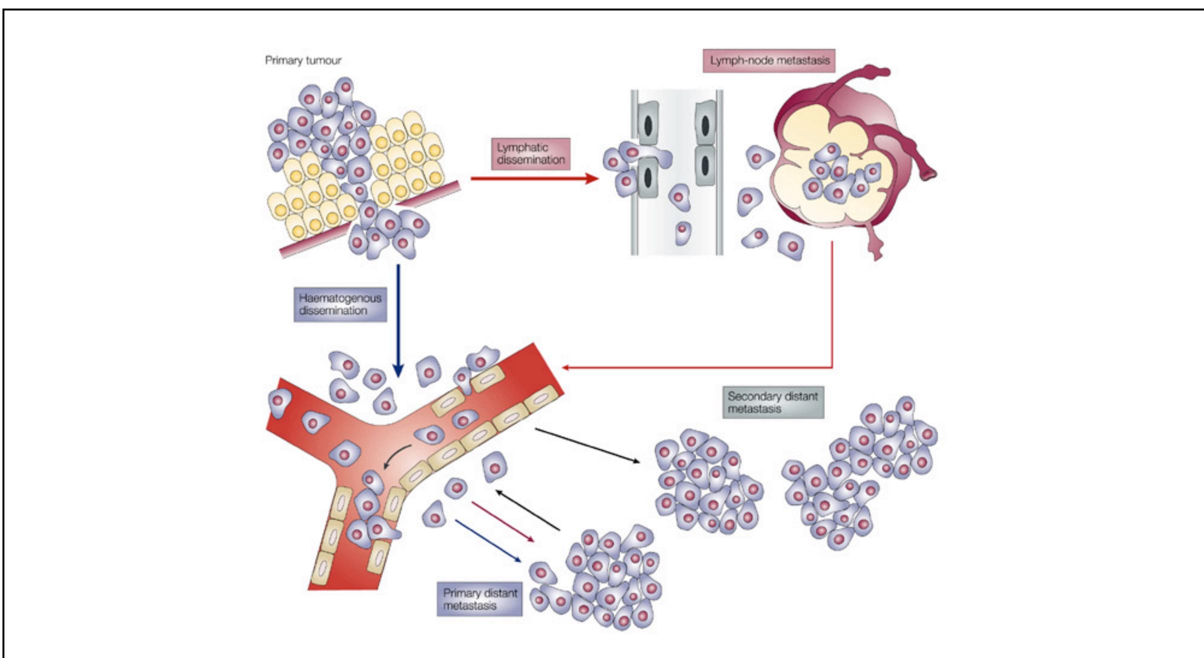

Fig. 2: Two routes of metastasis formation
Tumour cells disseminate from the primary tumour and invade adjacent tissues. After intravasation they use the blood or the lymphatic system for transportation. Via lymphatic dissemination the cells are transported to lymph nodes, where metastases are formed. Subsequently, tumour cells can enter the blood system through this route as well. Circulating tumour cells in the blood have to attach to the endothelial vessel wall, extravasate and settle at a secondary site to form distant metastases. *Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Pantel and Brakenhoff, 2004).*

The first step during the metastatic process is the dissemination from the primary tumour mass. Adhesion to neighbouring cells and to the ECM is lowered by metastasising cells through modulating the functions of cell adhesion molecules (CAMs) of the immunoglobulin, cadherin and integrin families and other CAMs. For example, the function

of E-cadherin, a homotypic cell-cell interaction molecule ubiquitously expressed on all epithelial cells, is altered in many carcinomas during progression towards malignancy (Christofori and Semb, 1999). The loss of E-cadherin mediated cell-cell adhesion has been proposed to be a prerequisite for tumour cell invasion and metastasis (Birchmeier and Behrens, 1994). Indeed re-establishing functional E-cadherin expression could reverse the invasive phenotype of cultured tumour cells (Vleminckx et al., 1991; Birchmeier and Behrens, 1994). The loss of E-cadherin expression often is accompanied by *de novo* expression of motility promoting cadherins, like N-cadherin (Li and Herlyn, 2000; Tomita et al., 2000). Such a ‘cadherin switch’ occurs during normal embryonic development, when epithelial cells acquire a migratory phenotype (Hatta and Takeichi, 1986; Bendel-Stenzel et al., 2000). Another prominent example is N-CAM, the expression of which is switched from a highly to a poorly adhesive isoform in certain cancers (Johnson, 1991; Kaiser et al., 1996) or is generally downregulated in others (Fogar et al., 1997). Finally, cancer cells are able to adapt to a new environment and to modulate their interaction with matrix components, such as collagen, laminin etc., e.g. by changing their integrin repertoire. It has been reported that tumour cells can switch integrins in a way that attachment to proteolytically degraded ECM components and migration is favoured over tight adhesion to normal epithelial matrix. (Varner and Cheresh, 1996; Lukashev and Werb, 1998).

In order to invade adjacent tissues, tumour cells may express extracellular proteases by themselves or induce their expression in neighbouring stromal cells (Werb, 1997). Degradation of matrix components by proteases enables tumour cells to overcome restrictions due to cell-cell contacts and to invade neighbouring tissues. Prominent examples are the ‘matrix metalloproteases’ (MMPs), a family of secreted or transmembrane proteases capable of degrading various matrix components. Cancer cells can also deregulate MMP function by modulating the expression of specific inhibitors (TIMPs) or of molecules that activate MMPs through enzymatic cleavage.

Cancer cells use the blood circulation or the lymphatic system for transportation to secondary sites. To enter a blood vessel a cancer cell has to pass the endothelial cell layer, a process called intravasation. The majority of cells entering the blood stream will die due to mechanical stress or elimination by the immune system, a process called immune surveillance (Jakobisiak et al., 2003). Because lymphatic vessels consist of more loosely associated cells the lymphatic system is easier to enter (Alitalo and Carmeliet, 2002). Although vascular and lymphatic spread of tumour cells share the basic features, the molecular mechanisms might differ. Once transported to a secondary site, a cancer cell has to

rest and extravasate out of the circulation, which means that the cell has to build up adhesive properties again to be able to settle. In order to grow out and form a new tumour mass a cancer cell has to adapt to the new environment, build up cell-cell and cell-matrix contacts again and fulfil all the described capabilities to form a massive tumour.

In conclusion, the process of metastatic outgrowth above all requires dynamic adhesive properties of cancer cells, switching between a sessile and a motile state when needed. Recent evidence highlights that the crosstalk of a cancer cell and the microenvironment at both the primary and the secondary site plays a key role for the metastatic success of cancer cells (reviewed by Schedin and Elias, 2004).

1.2 The BSp73 cell system

In order to study late steps in tumour progression cell systems have been established, comprising two related cell lines, which exhibit differences in their metastatic potential. These cell lines may originate from the primary tumour and a metastatic lesion or may present two subclones derived from the same primary tumour. The BSp73 tumour cell system represents such a model. A stable cell line BSp73 was established from a spontaneous pancreatic adenocarcinoma of a BDX rat (Zoller et al., 1978). Several *in vivo* passages after subcutaneous application gave rise to tumours with different metastatic potential and finally led to the establishment of two subclones of BSp73. One clone, BSp73AS (AS), displaying only weak metastatic growth, whereas the other, BSp73ASML (ASML), exhibits a very high metastatic potential (Matzku et al., 1983). When injected into the footpad of syngenic rats, AS cells show strong local tumour growth but only reach the draining lymph nodes. In contrast, ASML cells display only very limited local tumour growth, rapidly spread through the lymphatic system and form miliary metastases in the lung, which will finally kill the animal. Different to AS cells which show a spread out epithelial morphology with long filopodia, ASML cells display a rounded cell shape without any visible spreading on substrate and a very limited ability to bind to matrix components, such as laminin, fibronectin and collagen. Moreover, these cells do not form a closed monolayer but rather detach from the substrate before reaching full confluency. (Matzku et al., 1985; Ben-Ze'ev et al., 1986; Raz et al., 1986).

Generation of monoclonal antibodies against membrane preparations of the two cell lines (Matzku et al., 1989) led to the identification of 5 differentially expressed surface molecules, namely a variant isoform of the cell adhesion molecule CD44 (Gunthert et al., 1991), the α6β4 integrin (Herlevsen et al., 2003), C4.4A, a molecule showing high homology to the uPA receptor (Rosel et al., 1998), the ephithelial cell adhesion molecule EpCAM (Wuerfel et al., 1999) and the tetraspanin D6.1A (Claas et al., 1998). Subsequent expression profiling of two sublines revealed several hundred differentially expressed genes (Nestl et a., 2001; Tarbe et al., 2002).

1.3 The cell-cell and cell-matrix adhesion molecule CD44

One of the first molecules being described to be involved in metastasis formation is CD44. The described BSp73 cell system was used to show that a single CD44 splice variant conferred metastatic potential to the otherwise locally growing BSp73AS tumour (Gunthert et al., 1991). CD44, first assigned as 'lymphocyte homing receptor' (Gallatin et al., 1983), is a broadly distributed single pass transmembrane glycoprotein involved in several physiological and pathological processes including development, wound healing, inflammation, haematopoiesis, immune response and tumour progression (Herrlich et al., 1998; Naor et al., 1997; Ponta et al, 2003). Posttranslational modification and excessive alternative splicing give rise to a diverse pool of proteins ranging between 80 and 200 kDa in size.

1.3.1 Structural properties of CD44

The CD44 gene locus spans about 50 kb of genomic DNA and is highly conserved among vertebrates (Naor et al., 1997). The corresponding pre mRNA consists of 20 exons, 12 of which can be regulated by alternative splicing (Gunthert et al., 1991; Screaton et al., 1992; Tolg et al., 1993; reviewed by Naor et al., 1997; Lesley et al., 1998). Theoretically, about 1000 putative different splice products can be generated in this way, but apparently not all combinations are expressed (Naor et al., 1997). The shortest CD44 isoform, called standard isoform (CD44s), with all variant exons excised, is expressed in nearly all vertebrate cells (Naor et al., 1997), while variant isoforms, named after the variant exons contained, show a

highly restricted expression pattern during embryonic development, in pathological backgrounds or in a cell type specific manner. Alternative splicing can be dynamically regulated depending on the activation state of the cell (Arch et al., 1992). Examples for cell type specific variants are CD44v8-v10, which is present on epithelial cells, while CD44v3-v10 is expressed by keratinocytes (Fig. 3).

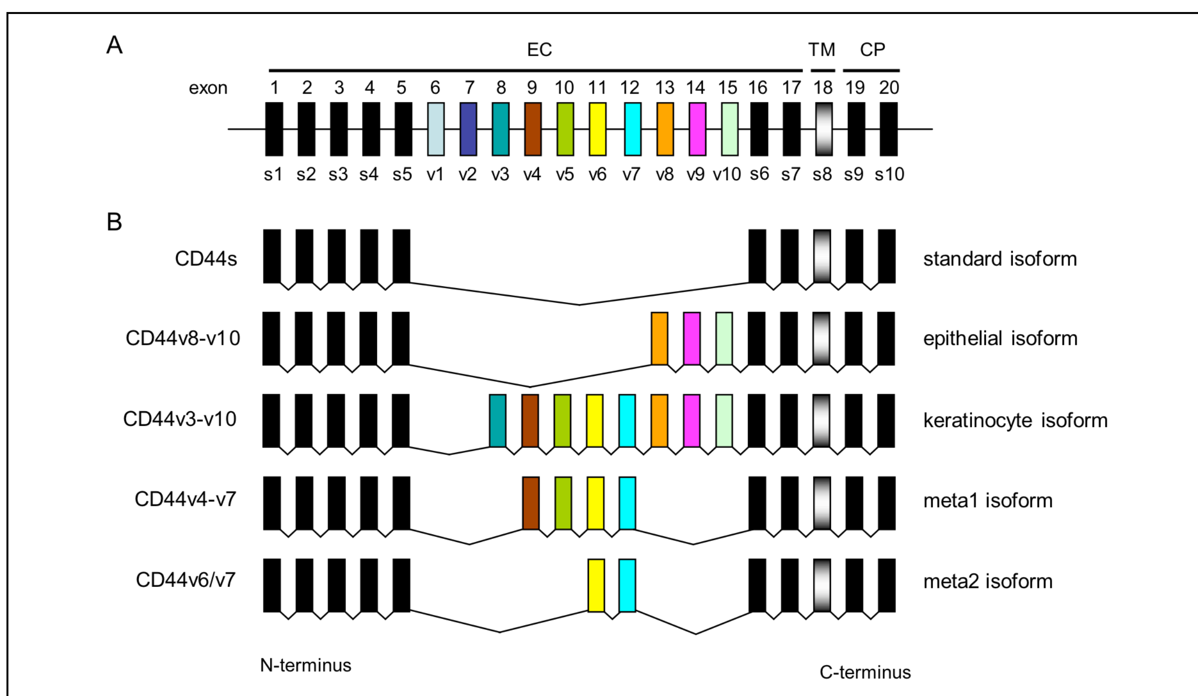

Fig. 3: Exon map of CD44 and examples for variant isoforms
A. Exon map of CD44 B. Examples for variant isoforms, named by the variant exons they contain. EC extracellular domain, TM transmembrane domain, CP cytoplasmic domain.

The CD44 protein consists of a large extracellular domain, made up of the amino-terminal part and a short membrane proximal stem structure, the transmembrane region and a cytoplasmic tail. Up to ten variant exon products can be inserted in the stem structure. In addition to this, a short version carrying a short cytoplasmic tail exists, which is only very rarely expressed (Goldstein and Butcher, 1990) (Fig. 4).

The extracellular domain of CD44 can be modified by N- and O-linked glycosylation and contains binding sites for hyaluronic acid (HA) and other glycosaminoglycans (GAGs) (Naor et al., 1997). Insertion of variant exons can lead to additional modifications. For example, variant exon 3 (v3) carries a site for heparan sulfate (HS) or chondroitin sulfate (CS) modifications (Bennett et al., 1995). Through its GAG binding sites CD44 can bind to GAG modified proteolycans such as versican (Kawashima et al., 2000), aggrecan (Fujimoto

et al., 2001) and serglycin (Toyama-Sorimachi et al., 1995). However, the functional relevance of this property is unclear. The extracellular domain of CD44 can be shed from the surface by proteolytic cleavage within the stem structure (Okamoto et al., 1999).

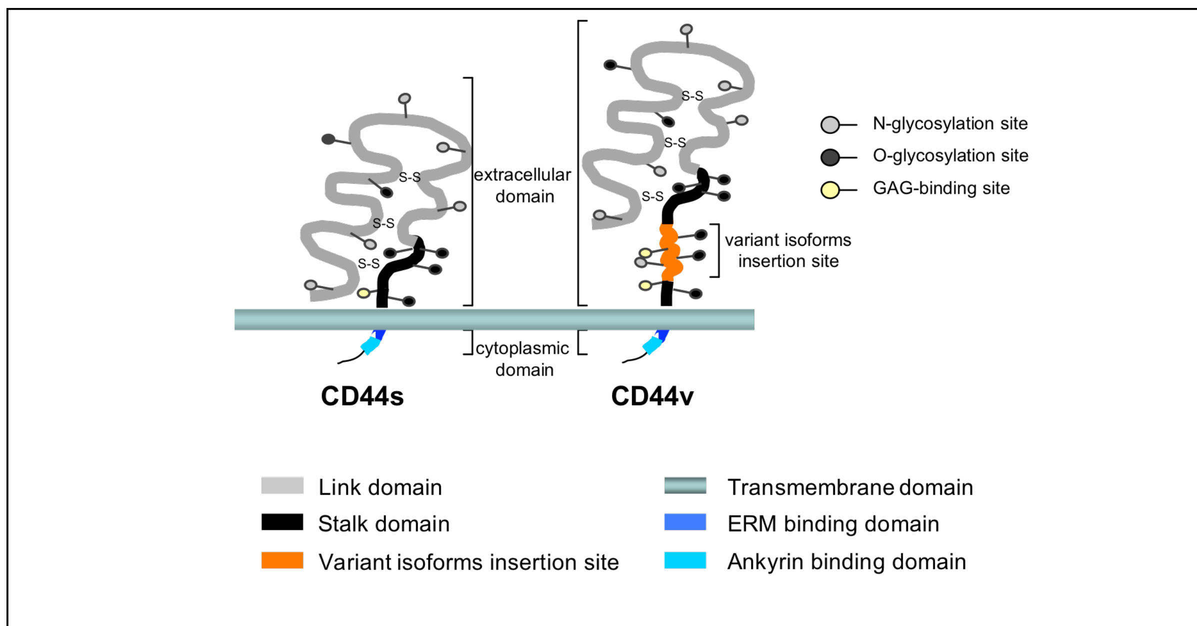

Fig. 4: Structure of the CD44 molecule
CD44 is a single pass transmembrane protein. The large extracellular domain carries binding sites for glycosaminoglycans, such as hyaluronic acid and sites for posttranslational modifications. 10 variable exons can be inserted into the stalk domain by alternative splicing. The short cytoplasmic tail can be linked to the cytoskeleton via ERM proteins and Ankyrin.

The transmembrane domain, encoded by exon 18 is supposed to be involved in CD44 oligomerization and localization in raft like membrane microdomains (Liu and Sy, 1997; Neame et al., 1995; Perschl et al., 1995), which are known to serve as signalling platforms. The cytoplasmic domain was shown to interact with a multitude of molecules and is important for linking CD44 to the cytoskeleton. The cytoplasmic tail of CD44 can be phosphorylated by protein kinase C (PKC), which influences its ability to interact with other proteins, as shown for ezrin (Legg et al., 2002). Upon shedding of the extracellular domain, the cytoplasmic part is cleaved and translocates to the nucleus where an effect on transcriptional regulation was demonstrated (Okamoto et al., 2001; reviewed by Nagano et al., 2004).

1.3.2 Different modes of interactions for CD44

A multitude of functions has been attributed to CD44 and its variants in different functional contexts, such as morphogenesis and organogenesis (reviewed by Knudson and Knudson, 1993), haematopoiesis (Ghaffari et al., 1999) and various immune functions, including homing and migration of lymphocytes and leukocyte activation and effector functions (reviewed in Naor et al., 1997; Pure and Cuff, 2001). The diversity of cellular processes influenced by CD44, e.g. growth regulation, survival, differentiation, adhesion and motility, raises the question how this can be achieved by a single molecule. Of course, one reason lies in the heterogeneity of the CD44 protein family with different variants each having unique characteristics. A second explanation might be the ability of CD44 to function in different ways. First as a ligand binding surface receptor, mainly by interacting with its principle ligand hyaluronic acid, second as a co-receptor for other surface molecules, modulating for example the signalling of associated growth factor receptors, and third through interactions with cytoplasmic molecules and as organizer of the actin cytoskeleton.

CD44 as a cell surface receptor

CD44 functions as the main hyaluronic acid receptor (Culty et al., 1990) but binding was also demonstrated for other components of the ECM, namely fibronectin, laminins and collagens (Turley and Moore, 1984) and also for cytokines like osteopontin and RANTES (Weber et al., 1996; Wolff et al., 1999). The corresponding binding site for HA is located in the standard part of the protein, but the affinity might be influenced by insertion of variant exons (Sleeman et al., 1996b) or by the state of glycosylation (Skelton et al., 1998). Moreover, binding to HA is not a constitutive ability of CD44 expressing cells but can be regulated from within the cell.

Hyaluronic acid belongs to the family of glycosaminoglycans, but different from all other GAGs, HA does not possess a protein component. Instead it displays a simple structure as a large polysaccharide, exclusively composed of repeating disaccharides of glucoronic acid and N-acetylglucosamine. Under physiological conditions HA consists of 2000 to 2500 disaccharides, corresponding to a molecular mass of 10^6-10^7 Da and a polymer length of 2-25μm. HA is a major component of the extracellular and pericellular matrix (Lee and Spicer, 2000) and due to its hygroscopic characteristic it plays an important role in tissue homeostasis and biomechanical integrity. The role of CD44 in many physiological and

pathological processes is based on its interaction with HA. By binding to its ligand, CD44 can mediate adhesion to and migration on HA rich matrices and these processes can be regulated by modulation of the binding affinity or by enzymatic cleavage of the extracellular portion of CD44 (Okamoto et al., 1999-2). Moreover, CD44 expression can influence the synthesis and endocytosis of HA (Culty et al., 1992; Hua et al., 1993).

CD44 was shown to recruit and regulate the activity of proteases on the cell surface. For example, MMP9 (Bourguignon et al., 1998) and MMP7 (Yu et al., 2002) were demonstrated to associate with CD44 and their function was dependent on this co-localization. In addition, growth factors and cytokines are captured by CD44, which can be variant specific as it is discussed for osteopontin (Katagiri et al., 1999). Growth factors described to bind to CD44 are 'hepatocyte growth factor'/'scatter factor' (HGF/SF), 'basic fibroblast growth factor' (bFGF) and 'heparin-binding factor' (Sherman et al., 1998; van der Voort et al., 1999; Jones et al., 2000). The enrichment of soluble molecules on the surface by CD44 can influence outside in signalling either directly via binding to CD44 or indirectly by regulating the binding to other receptors.

CD44 as a 'co-receptor' for other transmembrane proteins

Lacking a catalytic domain itself, CD44 can act as a co-receptor for protein tyrosine kinases (PTKs) like growth factor receptors. For example, CD44v6 has been demonstrated in several cell lines to be essential for proper binding of HGF to its cognate receptor c-Met/HGF-R through complex formation (Orian-Rousseau et al., 2002). A second prominent example is the ErbB family of receptor tyrosine kinases, with some members showing dependence on complex formation with CD44 for proper activation (Bourguignon et al., 1997; Sherman et al., 2000). The nature of this co-receptor function might be due to clustering of receptor subunits and stabilisation of receptor dimers or, as in the case of ErbB4, by supporting activation of the ligand via proteolytic cleavage through associated MMPs as mentioned above (Yu et al., 2002). Given the heterogeneity of the CD44 protein family, functioning as a co-receptor could explain the ability of CD44 to modulate several different signalling circuits, without any direct signal transfer through CD44 itself ever being demonstrated. CD44 also associates with other transmembrane proteins without catalytic activity, for instance tetraspanins and other adhesion molecules such as integrins or EpCAM (Schmidt et al., 2004; Ladwein et al., 2005).

CD44 as an associating molecule with cytoplasmic proteins and as organizer of the actin cytoskeleton

Several intracellular molecules were shown to associate with the cytoplasmic tail of CD44. Importantly, CD44 can be crosslinked to the actin cytoskeleton via binding to ankyrin and members of the ERM proteins (for ezrin, radixin and moesin) (Tsukita et al., 1994; Bourguignon and Jin 1995). ERM proteins are involved in the regulation of cell shape, cell migration and protein resorting in the plasma membrane (Bretscher et al., 2002; Gautreau et al., 2002) and CD44 was demonstrated to influence these processes through interaction with ERM proteins. Even though, the precise mechanism, e.g. if this leads to actin contraction, polymerization or depolymerization is not clear. The binding affinity for ERM proteins is tightly regulated and seems to be higher for variant CD44 than for the standard isoform (Tsukita et al, 1994). Other associated molecules include cytoplasmic kinases like Src, PKC, LCK and Fyn and the guanine nucleotide exchange factors TIAM1 and VAV2 (reviewed by Naor et al., 1997; Bourguignon et al., 2000; Bourguignon et al., 2001a+b).

1.3.3 Physiological and pathological functions ascribed to CD44

As mentioned above, the physiological roles of CD44 are surprisingly diverse. The fact that CD44 knock-out (k.o.) mice are viable and show only a very mild phenotype with regard to haematopoiesis as well as lymphocyte activation and migration (Schmits et al., 1997; Protin et al., 1999) argues for other molecules being able to compensate for the loss of CD44 during embryonic development. However, antibody blockade led to a retardation of development (Zoller et al., 1997). This corresponds to the observation that the k.o. phenotype became more obvious, when CD44$^{-/-}$ animals were challenged, for example by infection with pathogens or by artificial induction of autoimmune diseases and proved a role for CD44 in the immune system. The same holds true in variant specific k.o. mice (Wittig et al., 2000).

CD44 in development

Expression of CD44 during embryonic development has been investigated in several studies. However a defined role for CD44 was demonstrated only in a few cases, for example in axon guidance during the formation of the optic chiasm (Stretavan et al., 1994, 1995), during limb bud development (Sherman et al., 1998), and in uteric bud and mammary gland

development (Pohl et al., 2000). Expression of variant CD44 during embryogenesis is seen on most epithelial and haematopoietic cells (Wirth et al., 1993; Terpe et al, 1994; Weber et al., 1996), while the expression in adult animals is mainly restricted to the skin, the epithelium of the gut, some glands and subpopulations of the haematopoietic cells (Kennel et al., 1993; Wirth et al., 1993; Fox et al., 1994; Hirano et al., 1994). Several studies demonstrated an important function of CD44 for differentiation and proliferation of haematopoietic progenitor cells (Ghaffari et al., 1999).

CD44 in inflammation and leukocyte extravasation

Physiological functions of CD44 are best explored in haematopoietic cells and important roles for CD44 and HA were shown in inflammatory processes (Pure et al., 2001). The physiological importance of CD44 in this context was shown for example in autoimmune diseases like rheumatoid arthritis and many others (Brennan et al., 1997; Seiter et al., 1999; Stoop et al., 2001). During inflammation, leukocytes exit the circulation to enter into different tissues. The first step of this process, a loose attachment of the leukocytes to the vessel wall, termed 'rolling', is mediated by selectins on leukocytes binding to carbohydrate ligands on the endothelium. This primary interaction is followed by a secondary 'firm adhesion', mediated by integrins and subsequent extravasation (Albelda et al., 1994). There is increasing evidence that CD44 contributes to both, rolling and firm adhesion. CD44 on the surface of leukocytes was shown to mediate rolling on the vessel wall via interaction with endothelial HA (De Grendele et al., 1996/1997).

CD44 in pericellular matrix assembly

HA is a key player in the assembly of pericellular matrices through interactions with proteoglycans and other extracellular macromolecules. CD44 was shown to be the main HA anchoring molecule for these processes in chondrocytes, the main cartilage cell type (Knudson et al. 1996). Thereby, the presence of CD44 has implications for organizing the structure of the cartilage. Other cell types like fibroblasts are also able to form such a pericellular matrix (Hedman et al., 1979), which may be important for their locomotion (Turley et al., 1989). Although tumour cells usually do not synthesize their own pericellular matrix, they often have the ability to assemble one in the presence of exogenously added HA and aggregating proteoglycans (Knudson and Knudson, 1991).

1.3.4 CD44 in tumour progression

A role of CD44 for tumour progression is well documented (reviewed by Ponta et al., 1998; Naor et al., 2002; Marhaba and Zoller, 2004). The observation that variant CD44 is overexpressed in several metastatic tumours (Matsumura et al., 1992) raised the question whether CD44 might be predictive for metastasis formation. Several studies tried to define CD44 as prognostic marker, but correlations vary between different kinds of cancer, states of disease and the CD44 isoform being examined (Wielenga et al., 1993; Pals et al., 1997; Dall et al., 1994). However numerous studies have demonstrated that CD44s and especially variant CD44 is implicated in different aspects of tumour progression and particularly in metastatic spread. A direct evidence for this was demonstrated in a CD44 k.o. mouse model, showing unaltered primary tumour growth but inhibited sarcoma metastasis formation (Weber et al., 2002). Recently, CD44 could be identified as a key regulator for leukemic stem cell fate by blocking homing to the bone marrow (Jin et al., 2006). However, it has to be mentioned, that in prostate cancer cells overexpression of CD44s suppressed their metastatic capacity (Gao et al., 1998).

Deregulation of variant CD44 in several cancers is not surprising, taken into account, that aberrant alternative splicing is frequently seen in cancer cells (reviewed by Kalnina et al., 2005) and CD44 being most profoundly subjected to alternative splicing. This and the remarkable functional diversity of CD44 provide an explanation as to how one molecule can fulfil several different tasks of tumour progression. In fact, CD44 is described to influence most of the proposed acquired capabilities needed for successful tumour progression.

The interaction between CD44 and HA has been described to trigger proliferation of several tumour cell lines, such as melanoma cells (Ahrens et al., 2001), mammary carcinoma (Peterson et al., 2000), glioma (Akiyama et al., 2001) and malignant mesothelioma cells (Nasreen et al., 2002). On a molecular level CD44 was also demonstrated to promote tumour cell proliferation through its co-receptor function as mentioned above, by activating members of the ErbB receptor family (Bourguignon et al, 2001, Ghatak et al., 2005) and c-Met (Orian-Rousseau et al., 2002, Recio et al., 2003). In addition CD44v6 is able to directly induce proliferation by activating the MAP kinase pathway (Marhaba et al., 2005). In contrast, CD44 was also found to act as a tumour suppressor. Binding of CD44 to HA has been reported to inhibit cell growth during contact inhibition (Morrison et al., 2001) and induce terminal differentiation of myeloid leukemia cell lines (Charrad et al., 2002).

The influence of CD44 on apoptosis is even more controversial than on proliferation. In lymphoma and thymocytes CD44 engagement was shown to induce apoptosis through up-regulation of the proapoptotic molecule Bax and down-regulation of the antiapoptotic molecule Bcl-X_L (Guy et al., 2002). Proapoptotic effects of CD44 were also shown for dendritic cells and neutrophils (Yang et al., 2002; Takazoe et al., 2000). On the other hand, there are several examples for CD44 induced survival by suppressing the induction of apoptosis (Bates et al., 1998; Allouche et al., 2000). Survival-promoting functions of CD44 can be mediated by the PI3K-Akt pathway (Bates et al., 2001; Ghatak et al., 2002), or through upregulation of antiapoptotic molecules like Bcl-2 and Bcl-X_L (Khan et al., 2002; Marhaba et al., 2003). Moreover CD44 can also downregulate Fas expression, thereby inhibiting apoptosis in lung cancer cells (Yasuda et al., 2001), or inhibit Fas signalling through interaction with the receptor (Mielgo et al., 2006). Notably, these prosurvival effects are often attributed to variant CD44, like CD44v6 in colon carcinoma cells (Bates et al., 1998) or CD44v7 in lymphocytes (Wittig et al., 2000; Marhaba et al., 2003).

Factors known to stimulate vascularization are ‘basic fibroblast growth factor’ (bFGF), ‘vascular endothelial growth factor’ (VEGF) and cytokines such as ‘transforming growth factor β (TGFβ) (Pepper, 1997). MMP2 and MMP9 associated with CD44 were demonstrated to cleave the pro-form of TGFβ, thereby releasing the active cytokine and inducing angiogenesis (Yu and Stamenkovic, 1999, 2000). Other described angiogenic effects of CD44 are mainly due to its expression on endothelial cells rather than on tumour cells (Griffioen et al., 1997).

Finally, CD44 might influence the formation of metastatic lesions in several different ways. CD44-HA binding can influence the adhesive properties of tumour cells to the ECM. Modulation of this interaction can enhance the mobility of cancer cells during the metastatic process. For example HA-CD44 binding was shown to be important for glioma cell invasion and migration (Okada et al., 1996). Additionally, the loss of ECM contacts by cleavage of CD44 has been demonstrated (Okamoto et al., 1999). The loss of cell-matrix adhesion usually leads to growth arrest and cell death and CD44 was shown to be able to promote anchorage-independent growth, a prerequisite for invasion (Peterson et al., 2000; Ghatak et al., 2002).

The activation of cell surface MMPs by CD44 favours invasiveness of tumour cells, enabling them to migrate into adjacent tissues as demonstrated for MMP9 (Yu and Stamenkovic 1999, 2000). Migration of cells requires reorganization of the actin

cytoskeleton, which is under the control of Rho GTPases, like RhoA, Rac1 and Cdc42. HA binding to CD44 can activate Rac1 and induce lamellipodia formation and migration enabling invasive behaviour (Oliferenko et al., 2000; Bourguignon et al., 2000).

As already described in the context of inflammation, CD44v was shown to facilitate attachment of lymphoma cells to vessel walls through interaction with endothelial surface HA (Wallach-Dayan et al., 2001), facilitating subsequent firm adhesion and transmigration into the underlying tissue and favouring settlement of tumour cells at a secondary site. Indeed, a soluble form of CD44 competing with cell surface CD44 for HA binding reduced metastasis formation *in vivo* (Yu et al., 1997; Peterson et al., 2000).

In summary, published data clearly show that CD44 and especially variant CD44 play a crucial role in the course of tumour progression and metastasis formation.

1.4 RNA interference as a tool to study isoform specific gene functions

RNA interference (RNAi) depicts an evolutionary conserved mechanism, occurring in most eukaryotic organisms (Hannon, 2002), which may have evolved as defense system against viral and genetic parasites. Double stranded (ds) RNA molecules are processed into short RNA duplexes, directing sequence specific cleavage or translational repression of complementary messenger RNAs (reviewed by Meister and Tuschl, 2004) and are implicated in chromatin remodeling and transcriptional regulation (reviewed by Lippman and Martienssen, 2004). In addition to the growing importance of RNAi as a tool for manipulating gene expression, the endogenous triggers of the RNAi pathway, called microRNAs (miRNAs) recently attracted great interest and were shown to play a role during embryonic development and to be implicated in tumourigenesis as well (reviewed by O'Rourke et al., 2006; Esquela-Kerscher and Slack, 2006).

Upon the original observation that ds RNA triggers are far more efficient in gene silencing than single stranded (ss) antisense RNA molecules (Fire et al., 1998), RNAi became a popular tool for modulating gene expression in model systems like *C.elegans* and *Drosophila*. However the subsequent adaptation of the RNAi technique to the mammalian system (Elbashir et al., 2001) made RNAi one of the most profound discoveries of the last

decade and one of the most powerful techniques for studying gene function in a cell culture based system and probably even as therapeutic tool *in vivo*. Compared to other techniques, such as antisense technology or morpholino oligos, only RNAi enables splice variant specific down regulation of gene products even on a stable basis. Isoform specificity can be achieved by choosing exon specific target sequences and stable downregulation of the gene of interest can be realized through integration of expression cassettes, driving transcription of self complementary short hairpinRNAs (shRNAs), that are processed by the endogenous RNAi machinery into active small interfering RNAs (siRNAs) (Paddison et al., 2002; Brummelkamp et al., 2002). Thus, RNAi allows stable and isoform-specific loss-of-function studies, which were rarely practical by conventional k.o., due to the hyperploidity of many tumour cells.

1.5 Aims of the thesis

The aim of this work was to investigate the contribution of CD44v on the metastatic behaviour of ASML cells and to gain a better understanding of the role of variant CD44 during the lymphatic spread of tumour cells in general.

Due to its remarkable structural and functional heterogeneity, CD44 plays a pivotal role in several steps of tumour progression. First evidence for the importance of CD44 variants in metastasis formation was obtained in the rat pancreatic adenocarcinoma model BSp73, where two major splice variants expressed only by the highly metastatic ASML subline were identified as v4-v7 and v6/v7. Introduction of these isoforms conferred a metastatic phenotype to the otherwise only locally growing AS subline, exclusively expressing the standard CD44 isoform (Gunthert et al., 1991; Rudy et al., 1993). Moreover, application of antibodies targeting variant exon 6 retarded metastasis formation of ASML cells, providing a hint, that CD44v also plays a crucial role in the metastatic spread of these cells (Seiter et al., 1993). On the other hand, compared to ASML cells, AS cells display entirely different adhesion and migration characteristics. In addition to this, expression profiling revealed several hundred differentially expressed genes between these two sublines (Tarbe et al., 2002), including molecules known to play a role in cancer progression, such as c-Met and c-Myc.

In order to investigate specific functions of CD44v and the underlying molecular mechanisms in metastasis formation, a plasmid based RNAi system was used for creating

stable and variant specific CD44 knock-down cells of the highly metastatic carcinoma cell line BSp73ASML. The resulting phenotype was studied *in vitro* and *in vivo*, particularly emphasising interactions of CD44v with the microenvironment and the stimuli the tumour cell may receive by this crosstalk.

2. Results

A plasmid-based RNAi system (pSuper) was used to create stable and variant specific knock-down cells of the highly metastatic tumour cell line BSp73ASML (ASML), known to express CD44 variants at high levels. The plasmid drives expression of small hairpin RNAs, which are processed into functional siRNAs and eventually lead to sequence-specific down-regulation of the gene of interest. In addition, it carries a GFP reporter and a neomycin resistance for detection of transfected cells and selection of stable clones (Fig. 5). Constructs were designed on the premise to target the two most abundant CD44 variants expressed by ASML, namely CD44v4-v7 (meta1 isoform) and CD44v6/v7 (meta2 isoform), previously described to confer metastatic potential to the otherwise weakly metastatic sister subline BSp73AS (Rudy et al., 1993). Target sequences therefore lie within the variant exons contained in both isoforms. Construct 'v6' targets variant exon 6, 'v7' targets variant exon 7 and 'v6/v7' targets the border of exon v6 and v7. Constructs were cloned and verified by sequencing.

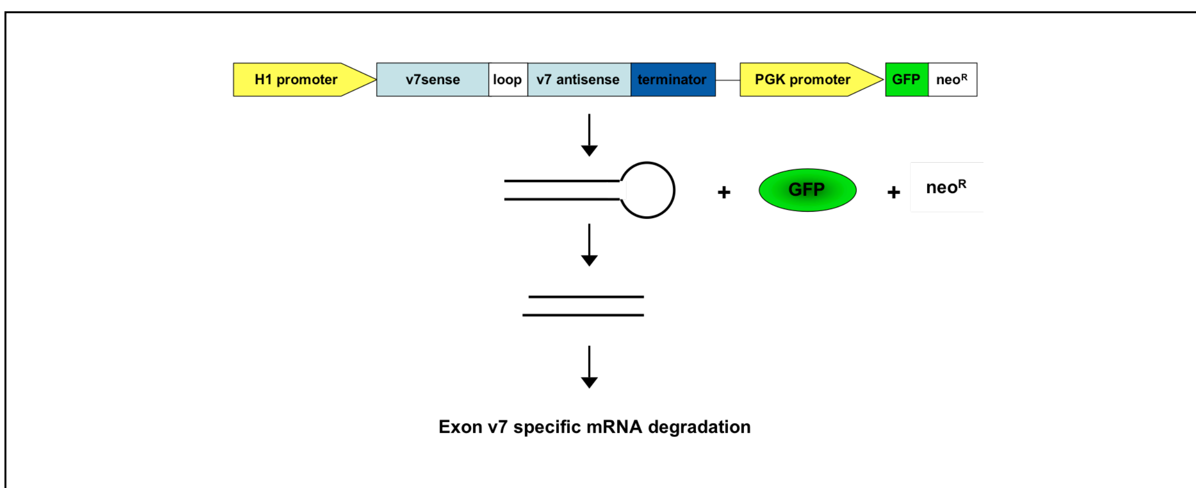

Fig. 5: Expression cassettes of 'pSuperGFPneo' carrying construct 'v7'
The self-complementary sequence of the transcript folds into a hairpin structure, which is processed by the endogenous RNAi machinery into an active siRNA. A GFP-reporter and a neomycin resistance are included in a second expression cassette.

2.1 Establishment of stable CD44vk.d. cell lines and rescue clones

2.1.1 RNAi construct evaluation by FACS and fluorescence microscopy

Efficiency in down-regulation of variant CD44 was monitored by FACS staining after transient transfection of ASML cells with three different pSuper-constructs and the empty vector (mock). Three days after transfection, cells were analyzed for CD44v expression by FACS staining using an antibody specific to variant exon 6 (A2.6). The mean intensity of GFP positive (transfected) cells was compared to GFP negative cells of the same pool and revealed functionality of the ‘v7’ and the ‘v6/v7’ constructs in down-regulating CD44variant expression on ASML cells. Table 1 shows the relative mean values of three experiments. While ‘mock’ transfection did not change CD44v expression and pSuper-v6 showed only weak down-regulation, transfection with pSuper-v7 reduced the mean intensity of CD44v by 55% and transfection with pSuper-v6/v7 resulted in a mean intensity reduced by 51%.

Tab. 1: Evaluation of pSuper constructs by FACS staining

construct	relative mean intensity for CD44v
pSuper-mock	108 ± 6%
pSuper-v6	86 ± 5%
pSuper-v7	45 ± 4%
pSuper-v6/7	49 ± 6%

FACS staining for CD44v6 (A2.6) after transient transfection with different pSuper constructs and the empty vector. Mean intensities of transfected (GFP positive) relative to untransfected cells are shown in percent.

Results were confirmed by immunofluorescence microscopy. ASML cells grown on coverslips were transfected with the three pSuper constructs and the empty vector and stained after three days for variant CD44 expression. GFP positive cells in pSuper-v7 and pSuper-v6/v7 transfections show reduced CD44 expression, while expression of other surface molecules like EpCAM is not affected (shown for pSuper-v7 in Fig. 6A). PSuper-v6 or mock transfected cells did not show a difference in CD44 expression (Fig. 6A, pSuper-v6 is not shown).

2.1.2 Establishment of stable CD44vk.d. clones by selection and recloning

By selection with G418 and two rounds of recloning three stable clones of pSuper-v7 transfected ASML cells were established that revealed strong down-regulation of variant CD44 on the protein level as shown by western blotting (Fig. 6B). CD44v levels are hardly detectable in the CD44v 'knock-down' (k.d.) clones, but unaffected in a stable 'mock' clone. EpCAM was used as an internal loading control. For unknown reasons no stable clones could be obtained with the pSuper-v6/7 construct.

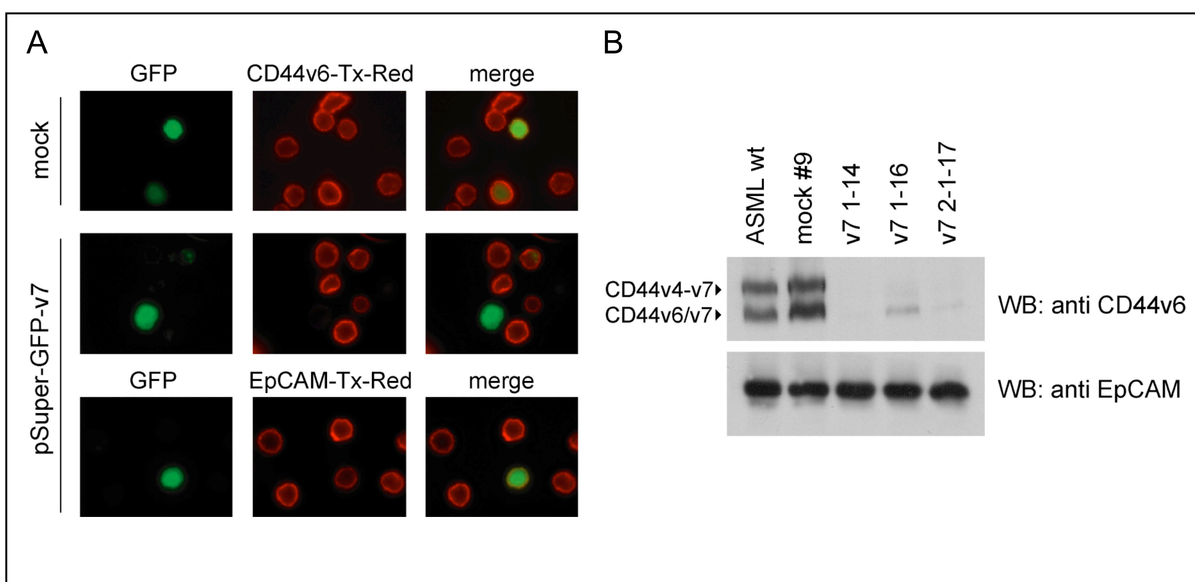

Fig. 6: CD44v expression of ASML cells is reduced after transfection with pSuper-v7 or the empty vector
A. Immunofluorescence staining for CD44v6 with A2.6 (upper panels) or EpCAM with D5.7 (lower panel). GFP positive cells display reduced CD44v expression only in the pSuper-v7 transfections (middle panel), while mock transfection has no influence on the expression level (upper panel). EpCAM staining is not affected (lower panel). B. Western blot analysis of stable clones. Upper panel: the two major CD44 variants are down-regulated in the three knock-down clones, but unaffected in the mock clone. EpCAM is used as an internal loading control (lower panel).

2.1.3 Restoring CD44 expression by introduction of mutated cDNAs

In order to control specificity of any phenotype arising in the k.d cells, 'rescue' clones with restored expression of one of the dominant CD44 variants were established. This was achieved by introduction of cDNAs coding for either CD44v4-v7 or CD44v6/v7, carrying four silent point mutations in the v7 target sequence, which should protect them from degradation (Fig.7A). Indeed co-transfecting 'HEK 293T' cells with the 'rescue' cDNAs together with the pSuper-v7 construct showed high CD44 expression, while expression of a wt CD44 cDNA was significantly affected by pSuper-v7 co-transfection (data not shown). One of the k.d. clones, ASMLv71-14, was used for transfections with the 'rescue' cDNAs and after selection and two rounds of recloning stable 'rescue' clones were established. Western blot analysis for CD44variant expression shows successful restoration of either CD44v4-v7 or CD44v6/v7 in the knock-down clone, although CD44v4-v7 expression was not restored to levels comparable to the wt situation. One mock clone is shown as control. EpCAM serves as a loading control (Fig. 7B).

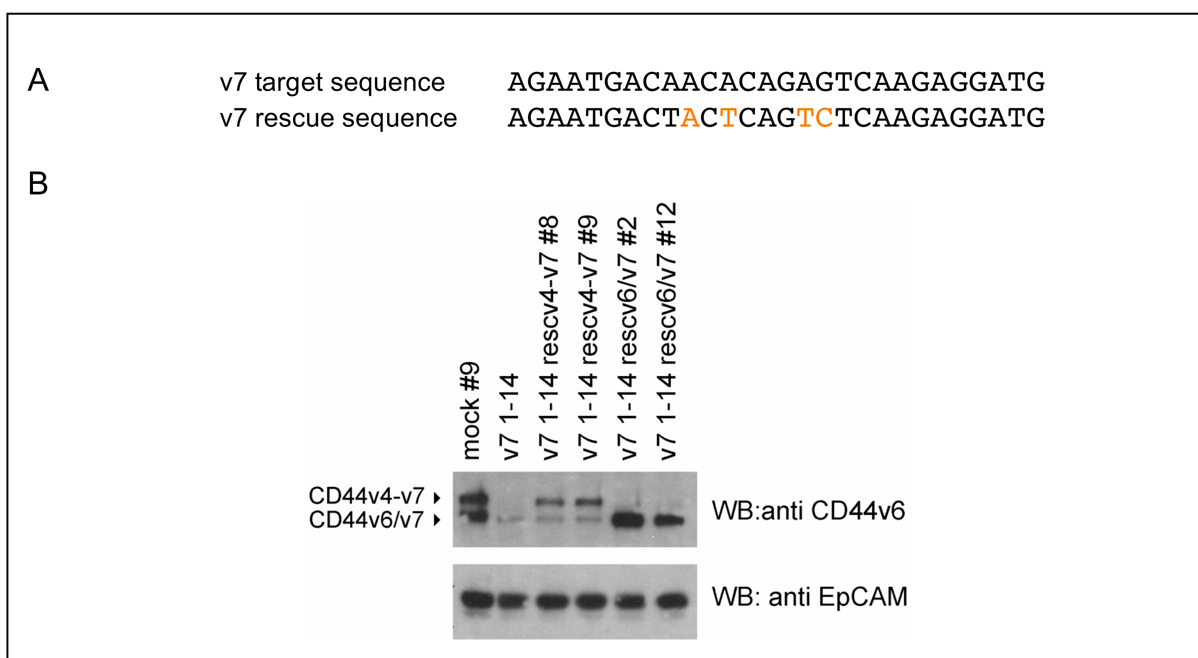

Fig. 7: Restoring CD44 expression by transfection with mutated cDNAs
Four silent mutations were introduced in the v7 target site of CD44v4-v7 and CD44v6/v7 cDNAs by PCR. The v71-14 clone was transfected with the mutated cDNAs and stable clones were established. A. v7 target and rescue sequence. Silent mutations are highlighted in red. B. Western blot analysis of stable clones, showing restored CD44v expression by A2.6 staining (upper panel). EpCAM is used as an internal loading control by D5.7 staining (lower panel).

2.2 Characterization of the knock-down cell lines *in vivo*

2.2.1 CD44vk.d. cells exhibit a reduced metastatic capacity *in vivo*

Intra-footpad injections (ifp) of BDX rats with ASMLwt, one mock and three k.d. clones were performed and the metastatic spread was monitored. The metastatic growth of ASML cells has been studied before (Matzku et al., 1983) and displays only little local tumour growth, but rapid spread through the whole lymphatic system with massive tumour burden in the proximal lymph nodes and miliar outgrowth of micro metastases in the lung, which eventually lead to the death of the animal. Animals were injected ifp with 10^6 cells and sacrificed after 50 days. Diameters of the primary tumour and lymph node metastases were measured and lungs were photographed and weighed. In addition, lung samples were analyzed by immunohistological staining. ASMLwt cells as well as the mock clone resembled the expected metastatic behavior, but the CD44vk.d. clones showed clearly less metastatic growth. Two k.d. clones (v71-14 and v72-1-17) displayed a greatly reduced over all tumour burden *in vivo*, while one clone (v71-16) grew as fast as the wt and the mock controls in the lymph nodes, while the metastatic settlement in the lung was clearly diminished in all three clones, with most animals revealing tumour free lungs and some only few metastatic nodules. In comparison, the wt- and mock-injected animals all displayed entirely metastatic lungs.

The metastatic burden of each lung was examined macroscopically (Fig. 8). The immense tumour burden in the lung of wt and mock treated animals is obvious by the size of the lungs that do not collapse, but are completely filled with tumour cells. Compared to this, the lungs of the CD44vk.d. clones appear normal or in the case of an v71-16-injected animal only moderately enlarged. The weight of the lungs gives a good indication for tumour burden (Tab. 2). Two animals injected with ASMLwt died before the end of the experiment, obviously due to the tumour burden of the lung. In addition, immunohistochemical analyses of sectioned lung samples were performed. C4.4A was used as a tumour marker in this case and again demonstrates the immense tumour burden in the lungs of wt and mock injected rats, while lungs of the k.d.-treated animals were tumour free or exhibited only few nodules as shown for a v71-16 lung. In this case also the stability of the RNAi effect throughout the *in vivo* experiment is demonstrated, as the v71-16 lung was unstained for CD44v6, while wt and mock tumour tissue is strongly stained (Fig. 8).

Tab. 2: CD44k.d. clones exhibit a reduced metastatic growth *in vivo*

injected construct-(animal#)	tumour burden				
	prim. tumour + lymph nodes		lung		
	tumour mass in cm^3	average	morphology	weight in g	average
untreated control-(1-1)	-	-	none	1.2	1.3±0.1
untreated control-(1-2)	-		none	1.3	
ASMLwt-(1-1)* †day38	5.5	6.9±2.1	miliary, confluent	5.3	5.1±1.1
ASMLwt-(1-2)* †day45	5.5		miliary, confluent	4.9	
ASMLwt-(2)	5.8		miliary, confluent	4.1	
ASMLwt-(3)	7.1		miliary, confluent	4.4	
ASMLwt-(4)	10.1		miliary, confluent	6.9	
mock9-(5-1)	6.6	6.9±2.6	miliary, confluent	6.7	4.8±1.8
mock9-(5-2)	3.7		miliary, confluent	2.4	
mock9-(6-1)	7.2		miliary, confluent	6.6	
mock9-(6-2)	8.0		miliary, confluent	5.5	
mock9-(7-1)	4.7		miliary, confluent	2.8	
mock-(7-2)	11.2		miliary, confluent	4.8	
v71-14-(11)	2.7	3.5±1.0	none	1.2	1.3±0.4
v71-14-(12-1)	3.2		none	1.1	
v71-14-(12-2)	3.5		none	1.3	
v71-14-(13-1)	4.2		none	1.3	
v71-14-(13-2)	5.0		none	1.9	
v71-14-(14-2)	2.1		none	0.9	
v72-1-17-(15-1)	1.0	2.3±1.4	miliary, few	1.6	1.5±0.1
v72-1-17-(15-2)	2.2		none	1.3	
v72-1-17-(16-1)	1.0		none	1.4	
v72-1-17-(16-2)	2.0		none	1.4	
v72-1-17-(17)	2.7		none	1.5	
v72-1-17-(18)	4.7		none	1.5	
v71-16-(8-1)	6.5	6.8±2.5	miliary, few	2.3	2.2±0.9
v71-16-(8-2)	8.6		miliary, few	2.2	
v71-16-(9-1)	10.0		miliary, multiple	3.8	
v71-16-(9-2)	7.5		none	1.8	
v71-16-(10-1)	3.5		none	1.2	
v71-16-(10-2)	4.5		none	1.7	

In vivo metastasis assay #1. Ifp injections of BDX rats with ASMLwt, mock and CD44vk.d. clones. Animals were sacrificed and dissected after 50 days. The tumour burden is given as sum of primary tumour and lymph nodes. The mean for each group is shown. Lungs were macroscopically examined and the weight is given as an indication for tumour burden of the lung. Two ASMLwt animals marked with asterisks (*) died within the course of the experiment.

The experiment was repeated with the rescue clones, displaying restored CD44v expression. One mock clone, one knock-down clone (v7 1-14) and one clone of each rescue construct were injected as before and animals were killed after 60 days and analyzed as described above. Again the mock-treated animals displayed massive tumour burden with miliary lungs, while the k.d. clone showed less metastasis formation without any tumour growth in the lungs. For unknown reasons the v4-v7 rescue clone did not grow *in vivo*, but four out of six animals injected with the CD44v6/v7 rescue clone displayed enhanced metastatic growth in the lymph nodes and five of six animals revealed also settlement in the lung, without reaching the massive tumour burden of the wt (Tab. 3). Immunohistology on

lung samples were carried out as before and confirmed the macroscopical examination (Fig. 8).

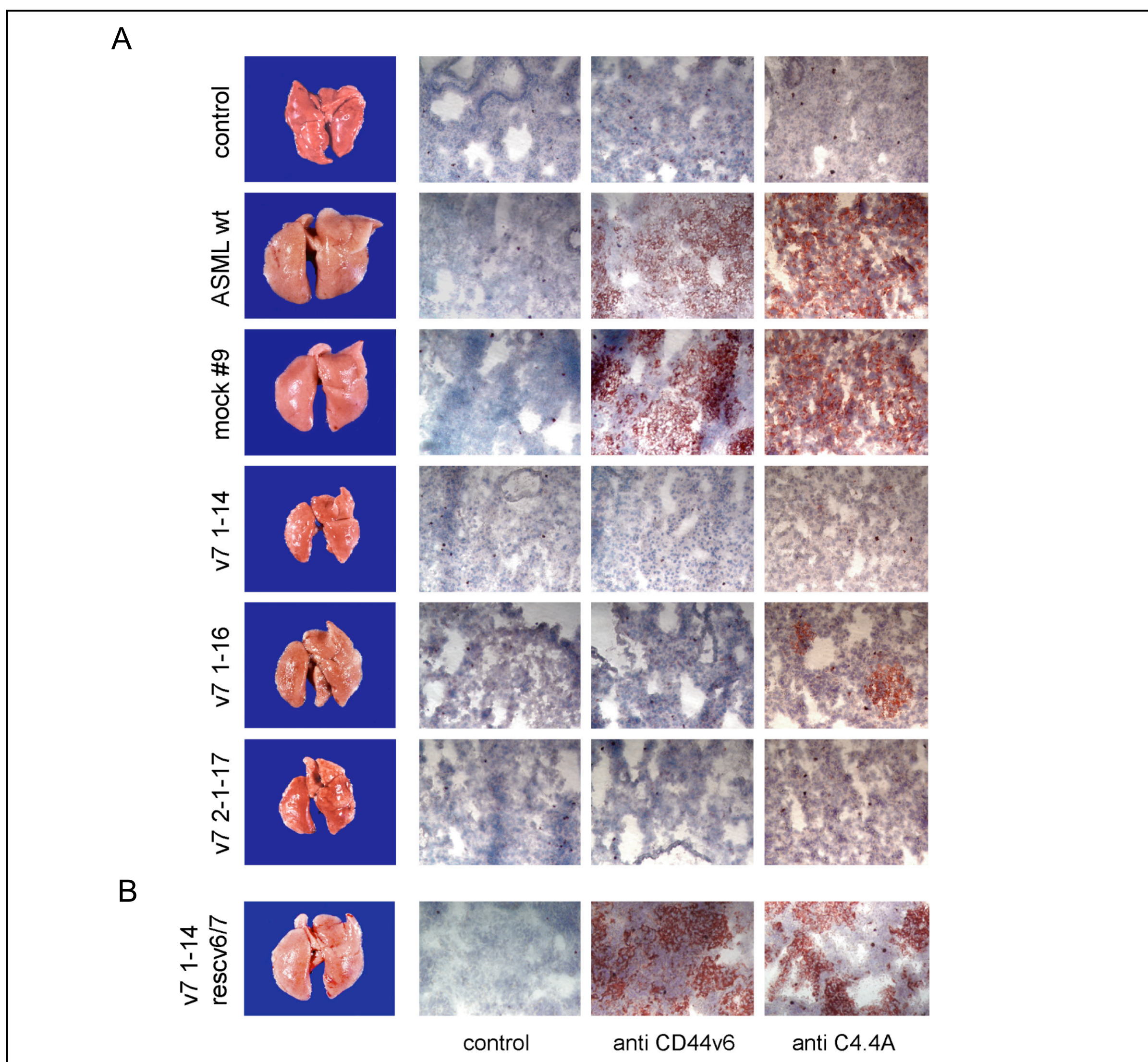

Fig. 8: CD44vk.d. cells exhibit a reduced settlement in the lung
Ifp injections of BDX rats. Animals were killed, dissected and lungs were analyzed. Macroscopic photographs of whole lungs and imunohistological analysis of sectioned lung samples, stained for CD44v6 (A2.6) and C4.4A (C4.4) as a tumour marker are shown. A. *In vivo* metastasis assay #1, showing ASMLwt, mock and CD44vk.d. clones. B. *In vivo* metastasis assay #2, showing one rescue clone (v71-14rescv6/7).

The stability of the k.d. and the restored CD44v expression was reconfirmed by re-cultivation of tumour cells from the lungs or lymph nodes of the injected clones. After lysis, SDS-PAGE and western blotting, blots were stained for CD44v6 (A2.6) and EpCAM (D5.7) as loading control. This assay clearly demonstrates the stable down-regulation of variant CD44 in the established clones and the restored CD44v6/v7 expression in the rescue clone (Fig. 9).

Tab. 3: Restored CD44variant expression is able to rescue the metastatic capacity of ASML in part

injected construct-(animal)	tumour burden				
	prim. tumour + lymph nodes		lung		
	tumour mass in cm^3	average	morphology	weight in g	average
mock9-(1-1)	9.0	7.0±1.9	miliary, confluent	8.7	6.0±1.6
mock9-(1-2)	5.7		miliary, confluent	6.2	
mock9-(2-1)	4.5		miliary, confluent	4.4	
mock9-(13)	6.3		miliary, confluent	4.8	
mock9-(15-1)	8.7		miliary, confluent	5.2	
mock9-(14-2)	7.2		miliary, confluent	6.5	
v71-14-(4-1)	4.5	3.3±1.0	none	1.5	1.3±0.2
v71-14-(4-2)	2.3		none	1.3	
v71-14-(3-1)	3.7		none	1.4	
v71-14-(3-2)	4.1		none	1.2	
v71-14-(5)	2.0		none	1.3	
v71-14-(14)	3.4		none	0.9	
v71-14rescv6/v7#12-(10-1)	10.3	6.2±4.0	miliary, confluent	3.0	2.1±0.6
v71-14rescv6/v7#12-(11-1)	11.2		miliary, multiple	2.5	
v71-14rescv6/v7#12-(11-2)	6.3		miliary, few	2.1	
v71-14rescv6/v7#12-(12-1)	1		none	1.5	
v71-14rescv6/v7#12-(12-2)	2.2		miliary, few	1.8	
v71-14rescv6/v7#12-(17-1)	7.3		miliary, few	1.6	

In vivo metastasis assay #2. Ifp injections of BDX rats with mock, one CD44vk.d. clone (v71-14) and two v71-14 rescue clones. Animals were killed and dissected after 60 days and tumour burden is given as sum of primary tumour and lymph nodes. The mean value for each group is shown. Lungs were macroscopically examined and the weight is given as an indication for tumour burden of the lung. The second rescue clone (v71-14rescv4-v7#8) did not grow *in vivo* and is not included in the table.

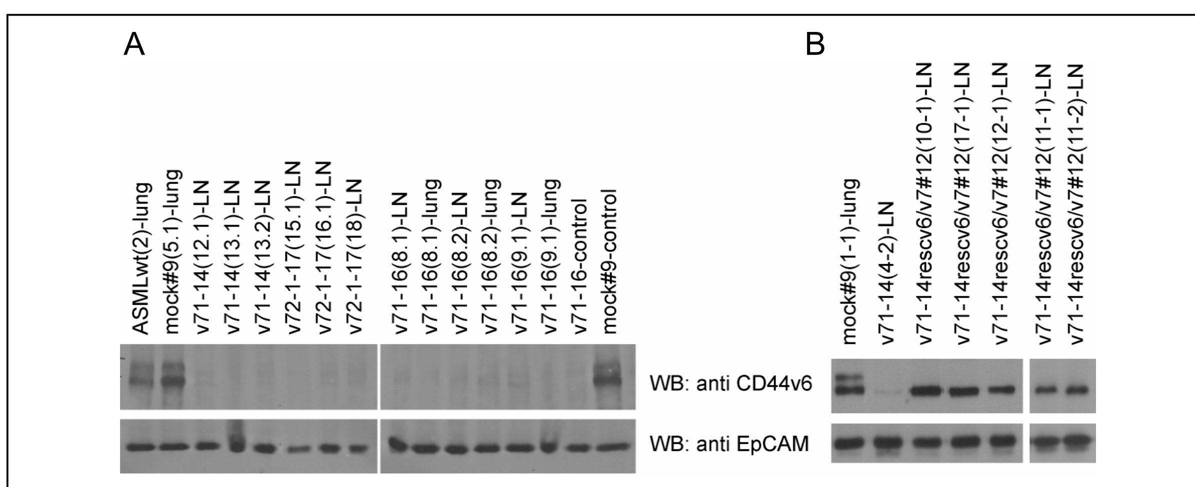

Fig. 9: Knocked down and restored expression of CD44v remained stable during the animal experiments

Western blot analysis on re-cultivated tumour cells from injected animals stained for CD44v6 (A2.6) and EpCAM (D5.7) as loading control. A. All tested k.d. tumour samples still show the same level of down-regulated CD44v. Cultured k.d. (v71-16) and mock cells are shown as control. B. All tested tumour samples of the rescue clones still show restored CD44v6/v7 expression. LN lymph node.

2.3 Characterization of knock-down cell lines *in vitro*

2.3.1 CD44vk.d. cells show no phenotypic changes

Cells were grown for 24 hours in 6-well plates. Microscopic analysis revealed no changes in cell shape or growth behavior. All clones display the same rounded cell shape without visible spreading. ASMLwt and CD44vk.d. cells do not form a dense monolayer with tight cell-cell contacts, but rather detach from the ground before reaching complete confluency (Fig. 10). The same accounts for the rescue clones. One rescue clone is shown as representative example.

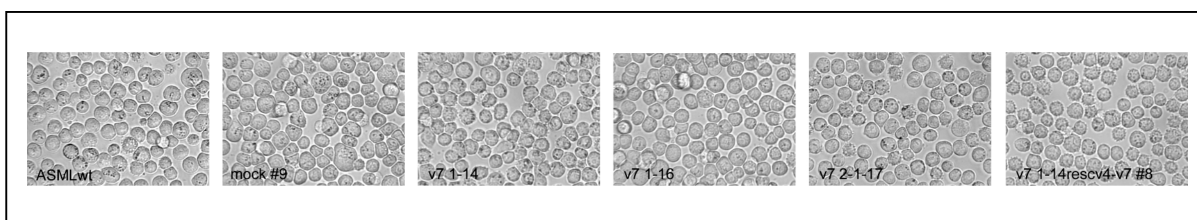

Fig. 10: The phenotype of CD44vk.d. clones is not changed
Microscopic analysis of wt ASML, mock, CD44vk.d. clones and one rescue clone, cultured for 24 hours.

2.3.2 CD44vk.d. cells show no altered growth behaviour

As the k.d. clones revealed different growth *in vivo*, the proliferative capacity was compared *in vitro*. Proliferation of wt and knock-down cells was monitored for three days, using either ^{3}H thymidine incorporation (data not shown) or staining with crystal violet. Both assays revealed no significant changes in proliferation rates between the wt and knock-down cells, irrespective of cells being grown in the presence of 10% FCS or under low serum conditions (0.5% FCS) (Fig. 11). Moreover, colony formation in soft agar, reflecting the ability of anchorage-independent growth, a hallmark of metastatic cells, did not show any significant changes. All clones revealed a high colony-forming efficacy of 90-95% (Tab. 4).

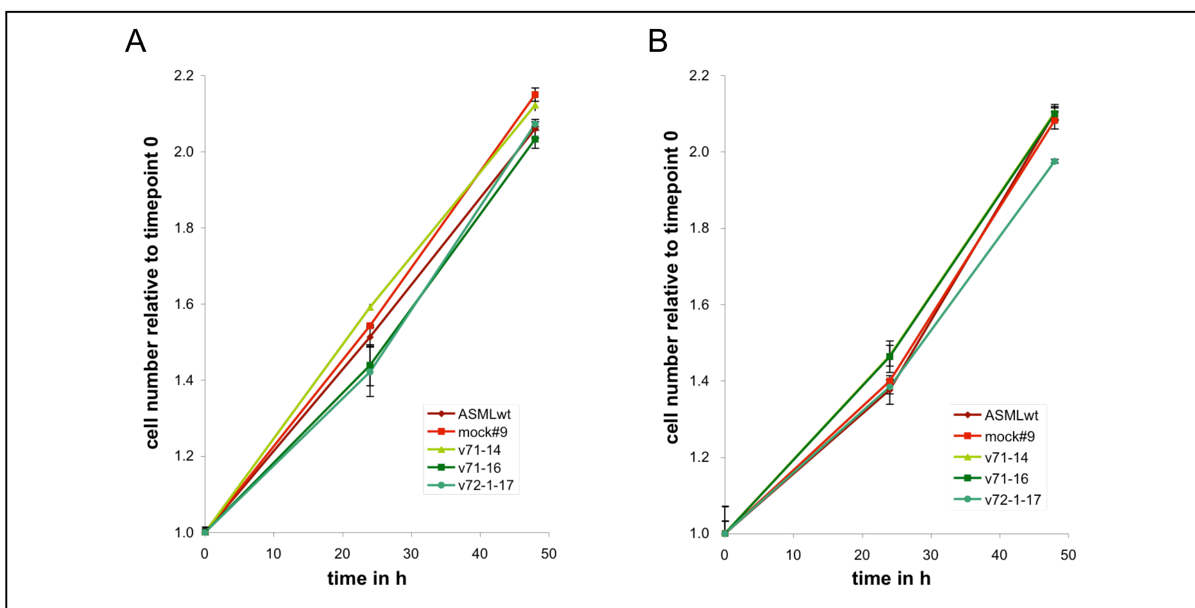

Fig. 11: Proliferation of CD44vk.d. clones is not altered
Proliferation was monitored for 48h and quantified by crystal violet staining. A. Proliferation in medium supplemented with 10% FCS. B. Proliferation in medium supplemented with 0.5% FCS.

Tab. 4: Soft agar colony formation of wt ASML, mock and CD44vk.d. clones

clone	colony forming efficacy
ASML wt	91% +/- 4%
mock #9	93% +/- 3%
v7 1-14	90% +/- 5%
v7 1-16	95% +/- 4%
v7 2-1-17	89% +/- 6%

100 and 1000 cells of ASMLwt, mock and CD44vk.d. clones were seeded in RPMI/10%FCS containing 0.5% agar. Colonies were counted after 4 weeks. The mean colony-forming efficacy is shown.

2.3.3 CD44vk.d. cells do not differ in MMP2 and MMP9 expression

In order to invade adjacent tissues, tumour cells have to degrade extracellular matrix barriers, which is often achieved by up-regulation of degrading enzymes like MMPs. To test if down-regulation of CD44 variants is accompanied by decreased MMP expression, MMP2 and MMP9 secretion into the supernatant was tested by a Gelatinease assay. Ten times concentrated conditioned cell culture supernatant of wt, mock and k.d. cells was subjected to SDS-PAGE, containing Gelatine as a substrate. After completion of the run gels were stained with Coomassie (Fig. 12). Enzymatic activity was visualized as unstained bands that reflect

the pro-forms of MMP2 and MMP9. No significant differences were observed between the clones.

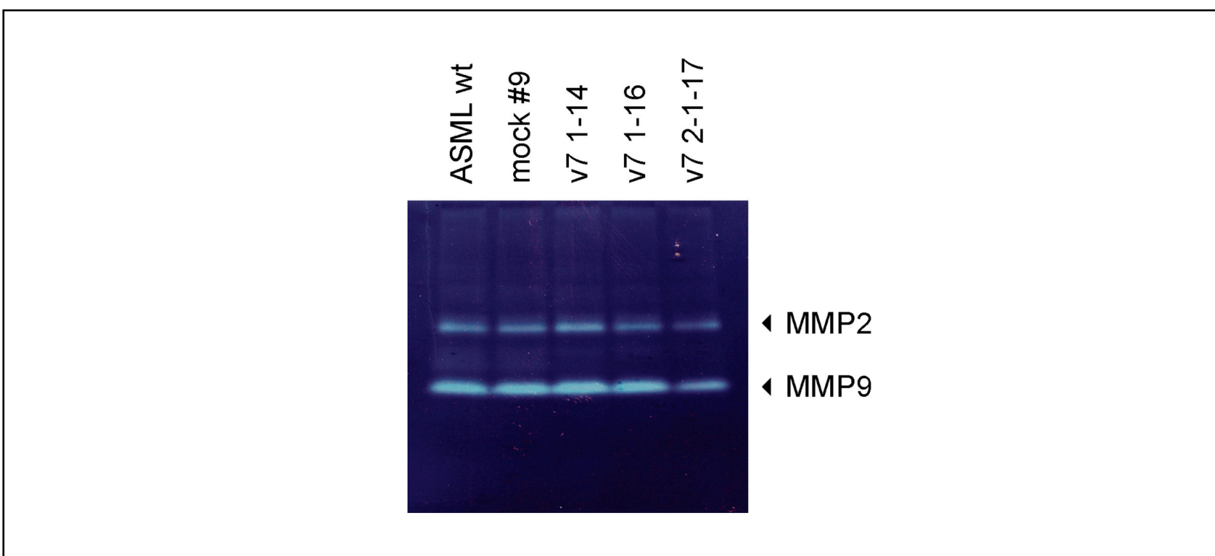

Fig. 12: MMP2 and MMP9 expression of wt ASML, mock and CD44vk.d. clones
Gelatine zymography of concentrated cell culture supernatants. Unstained bands correspond to Pro-MMP2 and Pro-MMP9 expression.

2.3.4 ASMLwt cells but not CD44vk.d. cells aggregate in stromal cell culture supernatant

As lymph nodal spread is significantly reduced in CD44vk.d. cells, it was tested, if ASML cells interact with lymph node stromal cells and if this interaction is impaired in the k.d. cells. Adhesion to monolayers of the lymph node stromal cell lines ‘ST-A4’ and ‘ST-B12’ or an immortalized lung fibroblast cell line did not reveal any affinity of ASML (data not shown). Instead it was noticed that ASML cells clumped and formed cell agglomerates when added to the stromal cells. This observation was tested subsequently with conditioned cell culture supernatant of the three mentioned stromal cell lines. Only wt and mock cells formed huge agglomerates in the supernatant within minutes, while CD44vk.d. cells did not clump at all or formed only very small cell clusters. The ability to form agglomerates was completely reestablished by the restored CD44v expression in the rescue clones (Fig. 13).

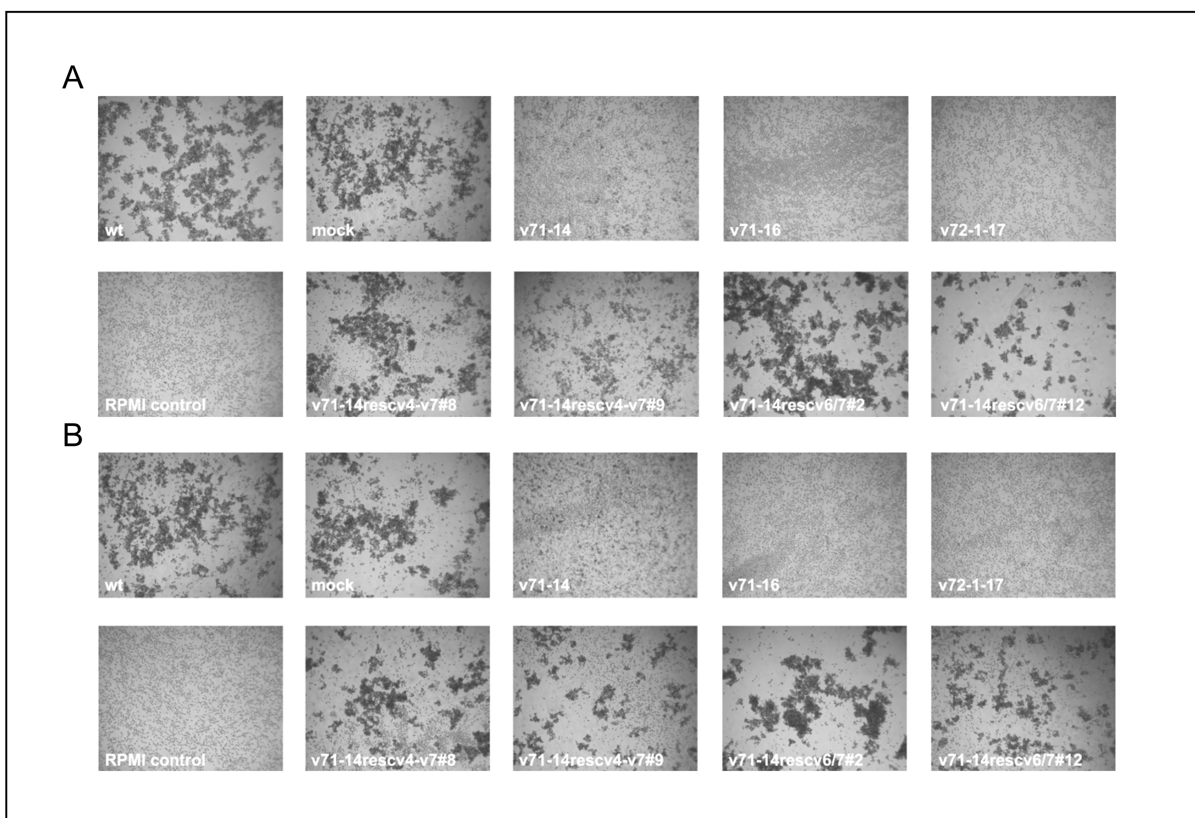

Fig. 13: Agglomerate formation in conditioned cell culture supernatant of stromal cells
Microscopic analysis of agglomeration of ASMLwt, mock, k.d. and rescue clones in conditioned cell culture supernatant of immortalized lung fibroblasts (A) and the lymph node stromal cell line ST-A4 (B). ASMLwt cells in RPMI medium are shown as control.

As hyaluronic acid (HA) is known to crosslink CD44 on the surface of cells and stromal cells are known to produce HA in large amounts, it was tested if the cell clumping was due to CD44 cross-bridging by HA in the supernatant. Supernatant was treated with hyaluronidase (1mg/ml) for 2 or 5 hours and agglomeration was monitored as before, heat-inactivated hyaluronidase served as a control. Indeed cell clumping was abrogated by hyaluronidase treatment in a time dependent manner (Fig. 14B). Likewise, addition of 1mg/ml HA to normal RPMI medium was able to induce agglomerate formation of ASML cells (Fig. 14A), demonstrating that the observed clustering is likely due to bridging of cell surface CD44 by HA.

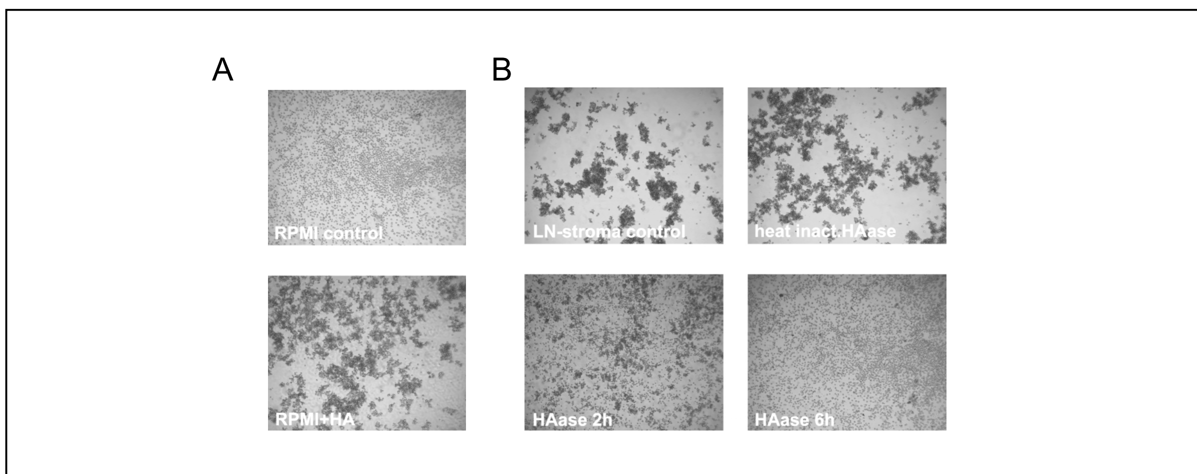

Fig. 14: Influence of hyaluronic acid on agglomeration of ASML cells
A. Addition of HA to normal RPMI medium is able to induce agglomeration of ASMLwt cells. B. Stromal cell culture supernatant was treated with hyaluronidase for 2 or 5 hours, which abrogates aggregation. Heat inactivated hyaluronidase was used as a control.

2.3.5 ASMLwt cells produce an adhesive matrix, which is impaired in the CD44vk.d.

ASML cells show a very limited ability to bind to matrix components, like fibronectin, laminin or collagen, while cultivated cells strongly attach to the plastic flasks. This feature is reduced in CD44vk.d. cells as noted by different sensitivities to trypsin treatment. To test for differences in adhesion, conditioned cell culture supernatant of ASMLwt cells was coated to plastic and used for adhesion assays. ASMLwt and CD44vk.d. cells show rapid adhesion to the coated supernatant, indicating that adhesion is not impaired in the k.d. (data not shown).

The supernatant of the knock-down cells was tested for adhesive properties after coating. Interestingly, a dramatic reduction was observed. Figure 15A shows reciprocal crisscross adhesion to matrices of wt cells, a mock clone and the three knock-down clones. All 5 clones adhere rapidly to the wt and mock derived matrix, while adhesion to the matrices produced by either of the knock-down clones is strongly impaired for all cells. To test if adhesion is induced by a soluble or deposited factor, cells were cultured in 24-well plates for 24 hours and detached by either trypsin or EDTA treatment. The plates were used for adhesion assays as described above. Only EDTA treated plates were able to promote adhesion (data not shown). Using this approach wt and knock-down cells were compared for their ability to deposit an adhesion promoting matrix. Indeed, the knock-down matrix was

clearly less capable of promoting adhesion than the wt matrix. However, the difference was less pronounced than for the conditioned supernatant (Fig. 15B). These results show that the loss of variant CD44 in ASML cells leads to an impaired ability to produce an adhesive matrix.

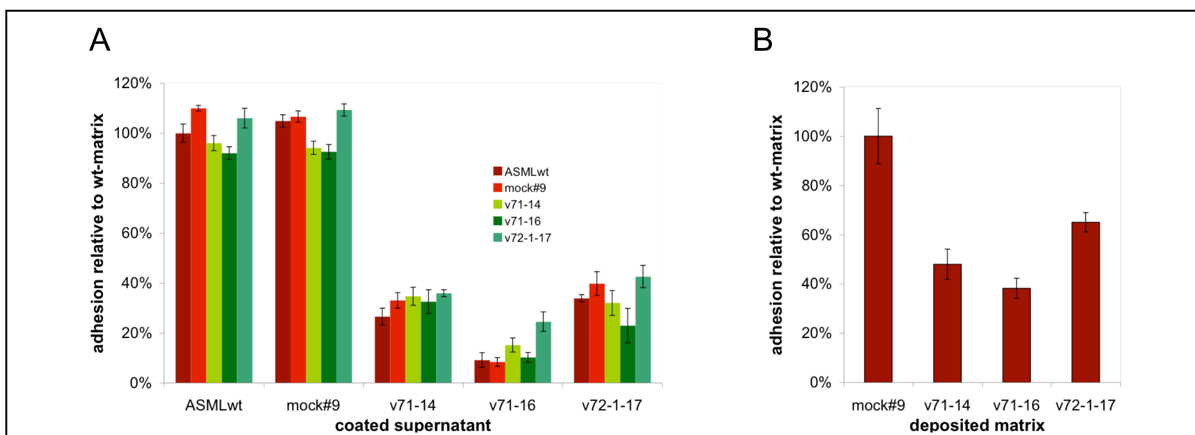

Fig. 15: Adhesion to coated supernatant and deposited matrix of wt ASML and k.d. cells
A. Adhesion assay on coated supernatant of wt ASML and k.d. clones. Relative adhesion of each line to the different matrices is shown. Adhesion of wt ASML to wt matrix was set to 100%. B. Adhesion of ASMLwt to deposited matrices of the different clones. Deposited matrix was prepared by removal of cells through EDTA treatment.

In order to investigate, whether this matrix defect is indeed due to the loss of CD44, the rescue clones displaying restored expression of CD44v4-v7 or CD44v6/v7 were used for matrix production and compared to the parental knock-down clone. As expected, restoration of either CD44 isoform was able to restore the matrix production significantly, but without reproducing the full adhesive properties of the wt matrix (Fig. 16). In summary, loss of variant CD44 in ASML cells leads to an impaired ability to generate an adhesion-promoting matrix, which is deposited on the plastic and secreted into the supernatant. Restoring CD44variant expression is able to restore this matrix production in part.

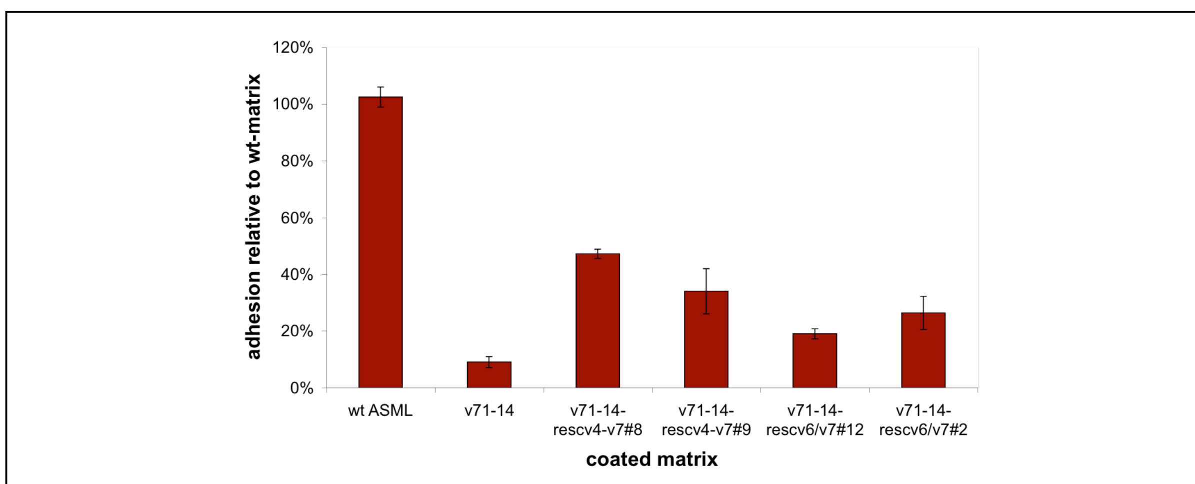

Fig. 16: Restoring CD44v expression rescues matrix production
Adhesion assay of ASMLwt cells to coated supernatant of ASMLwt, v71-14 and v71-14rescue clones. Adhesion of ASMLwt cells to wt matrix is set to 100%.

2.3.5.1 Adhesion promoting components are secreted

To verify that the components mediating adhesion are secreted factors, and not present on membrane particles, like exosomes or membrane fragments, ultracentrifugation (100000*g*) was performed, which should precipitate all membrane particles present in the supernatant. The resuspended pellet and the supernatant were tested for adhesive properties after coating to plastic. Only the supernatant was able to promote adhesion of ASML cells (Fig. 17A). The tetraspanin D6.1A was used as exosomal marker (Wubbods et al., 2003) and was completely removed from the supernatant by centrifugation, while shed CD44 was present in the supernatant, demonstrating that the separation procedure was successful (Fig. 17B).

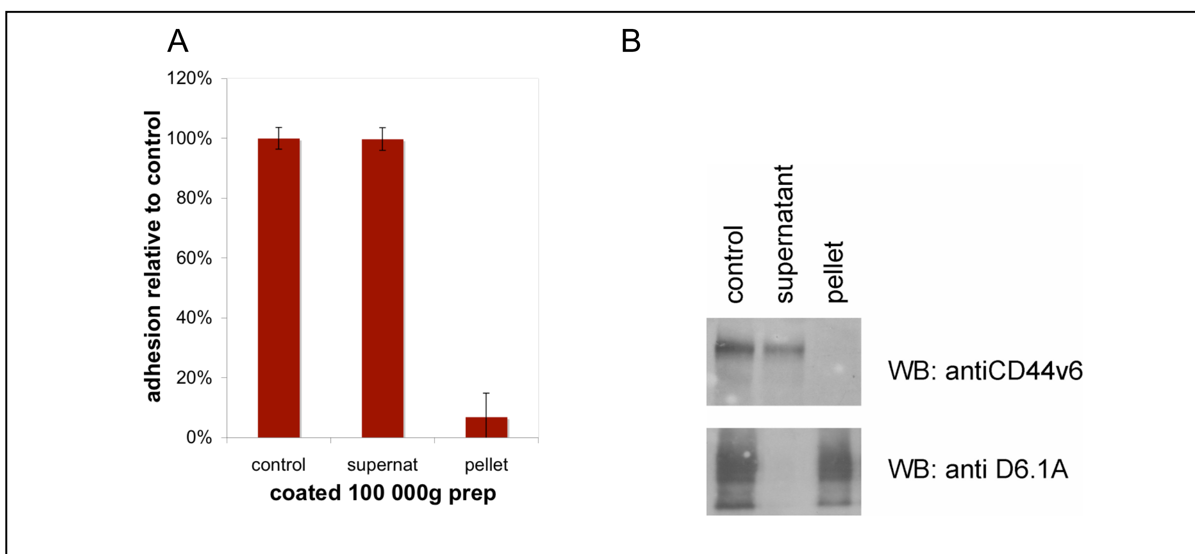

Fig. 17: Ultracentrifugation of cell culture supernatant
100000*g* centrifugation from concentrated supernatant of wt ASML A. Adhesion assay on coated supernatant and the resuspended pellet. B. Western blot control for shed CD44v6 (A2.6) and for D6.1A (D6.1) as an exosomal marker.

2.3.5.2 The secreted matrix contains HA, collagen and laminin

As CD44 is known to be involved in the assembly of pericellular matrices through anchoring hyaluronic acid, the matrix was tested for sensitivity to hyaluronidase treatment as well as for collagenase treatment. Conditioned supernatant was treated with different concentrations of hyaluronidase or collagenase either before or after coating to plastic and adhesion assays were performed as described. Heat-inactivated enzymes were used as controls (Fig. 18). While hyaluronidase treatment after coating did not alter the adhesive properties, it did destroy the ability to promote adhesion, when the supernatant was treated prior to coating (Fig. 18B). This indicates that HA is used as a scaffold for other matrix components rather than as adhesive substrate and implicates that adhesion itself is not mediated by CD44, which goes along with the finding that the k.d. clones do not display an impaired adhesion to the wt matrix. In contrast, collagenase treatment did abrogate the adhesive properties of the matrix irrespective if the supernatant was treated before or after coating (Fig. 18A). In summary, these results indicate that HA is needed for the assembly of other matrix components and that the rapid adhesion of ASML cells is mediated via binding to collagen.

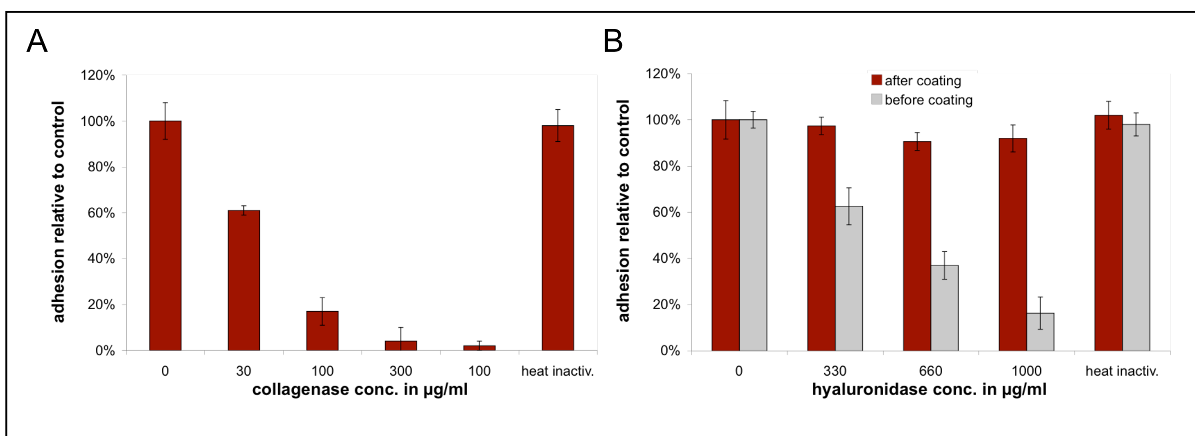

Fig. 18: Hyaluronidase and collagenase treatment disrupts the adhesive properties of the supernatant

Supernatant of wt ASML was treated with different concentrations of collagenase after coating (A) and hyaluronidase before or after coating (B). Adhesion assays were performed with ASMLwt cells as described earlier.

The adhesive portion of the secreted matrix is between 600 and 4000kDa in size

To further characterize the composition of the secreted matrix, conditioned supernatant was collected and concentrated 40 times through a 'vivaspin' column. The concentrate was size separated by gelfiltration using different pore sizes. Fractions were collected and used for coating to 24-well plates. The void volume of the column was defined by blue dextrane. Adhesion assays were performed and revealed that the fractions promoting adhesion were still within the void volume, when 'Superdex200' beads were used (Fig. 19A), but were within the separation range of 'CL6B' beads (Fig. 19B). This indicates that the components promoting adhesion are larger in size than roughly 600 kDa, (size exclusion of Superdex200) but smaller than 4000 kDa (size exclusion of CL6B). The adhesive fractions of wt cells after chromatography were compared to the corresponding fractions of the knock-down cells using silver staining. However, protein content was low in general and no visible differences could be observed (data not shown).

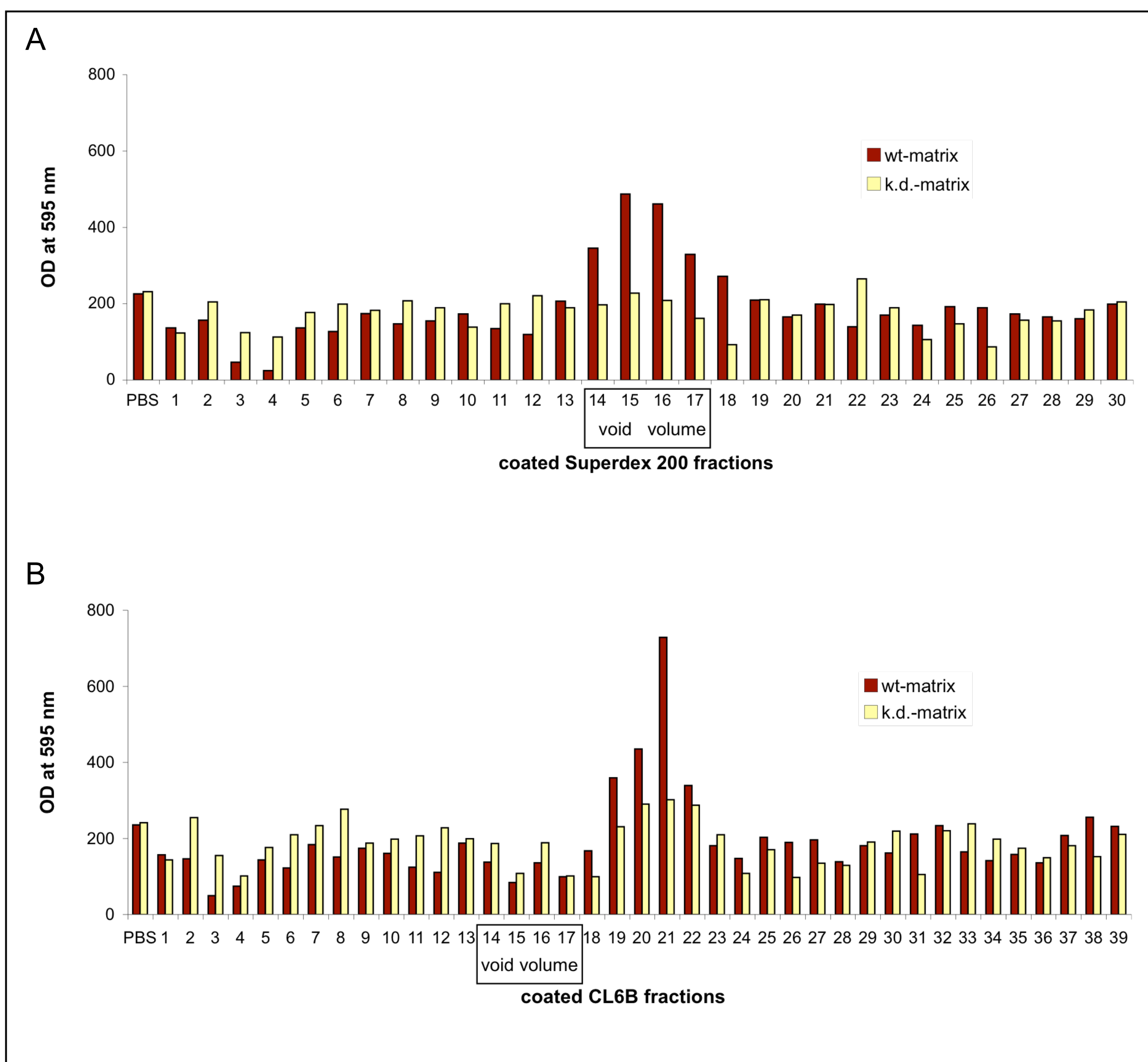

Fig. 19: Size exclusion chromatography of wt and k.d. supernatant
Concentrated supernatants of wt ASML and v71-16 k.d. cells were size fractionated by chromatography on Superdex200 (A) and CL6B (B) sepharose columns. Fractions were coated to plastic and used for adhesion assays. The void volume was defined by blue dextrane.

In order to identify components of the matrix mediating adhesion, CL6B fractions were subjected to SDS-PAGE and western blotting and probed with different antibodies. Shed CD44 was not present in the fractions promoting adhesion. In contrast, laminin was present, although not exclusively in the adhesive fractions and no difference in the laminin distribution was observed between the wt and the knock-down fractions (Fig. 20).

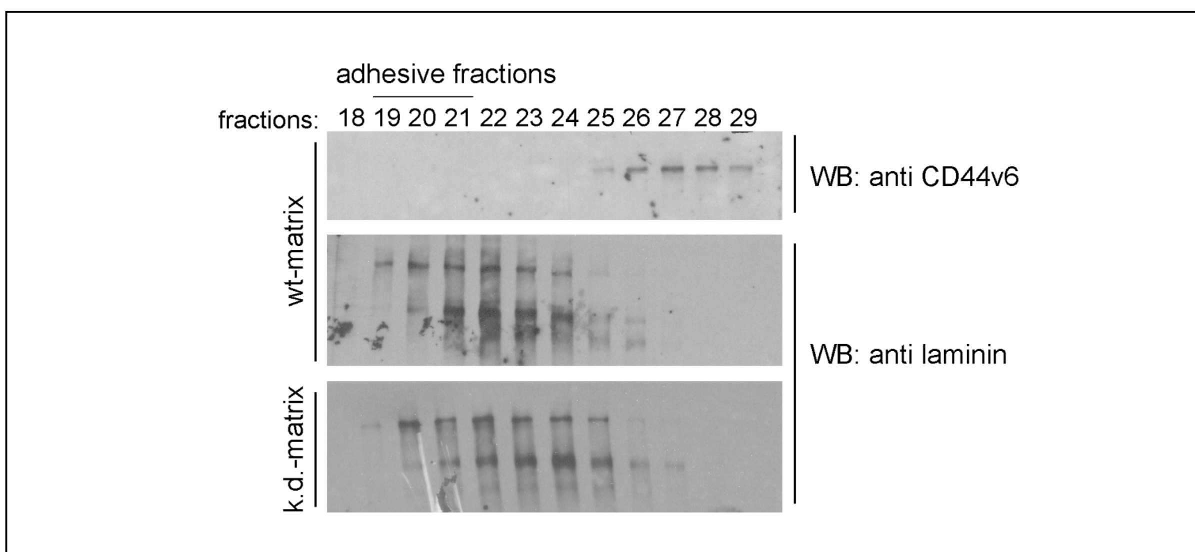

Fig. 20: Western blot analysis for CL6B-fractionated conditioned supernatant
Western blot analysis of fractionated supernatants. Concentrated supernatants of wt ASML and a CD44vk.d. clone (v71-16) were size fractionated by chromatography on CL6B sepharose columns. Fractions including the adhesive fractions (see Fig. 19) were subjected to SDS-PAGE and stained for CD44v6 (A2.6) and laminin (polyclonal serum recognizing several laminins).

2.3.5.3 Adhesion to the matrix is mediated by β1 integrin

The observation that trypsin treatment destroyed the deposited matrix but EDTA treatment left the matrix unaltered, together with the finding that collagen and not HA seems to be involved in the adhesion process suggests that adhesion of ASML to their own matrix could be mediated by integrins. In line with this, addition of EDTA during adhesion abrogated attachment of the cells, arguing for Ca^{2+} dependent adhesion (data not shown). To further test this hypothesis different antibodies were tested for their ability to block adhesion. For this purpose cells were pre-incubated with different integrin antibodies and adhesion was performed as before. Figure 21 shows that only a β1 integrin antibody blocked adhesion in a concentration dependent manner, while all other antibodies, including anti CD44, had no influence on adhesion. Anti α6β1 induced cell aggregation and could therefore not be used for blocking experiments. ASML cells do not express α1, α4 and α5 integrin chains (data not shown). Pre-incubation of the coated matrix with an anti laminin polyclonal serum (reacting with several laminins) did not interfere with adhesion. Therefore it seems likely that initial adhesion of ASML cells to their own matrix is mediated by a β1 integrin binding to matrix bound collagen, even though the specific type of collagen and the integrin α chain could not be identified.

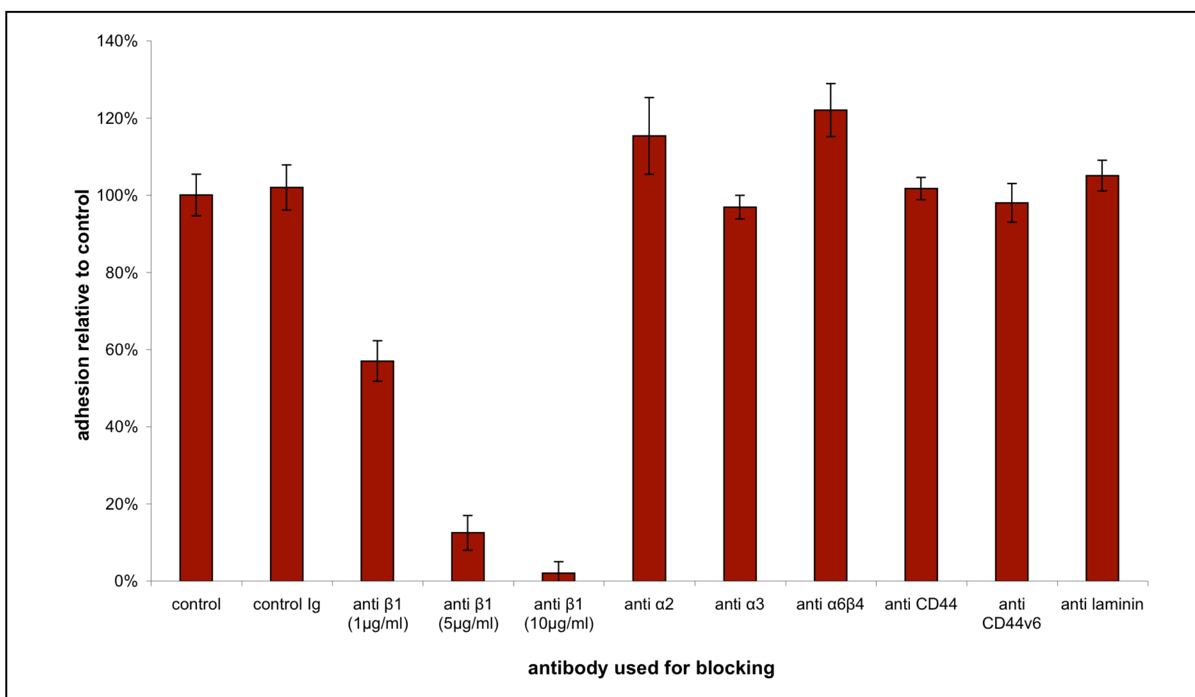

Fig. 21: Interfering with β1-integrin blocks adhesion to the secreted matrix
ASMLwt cells were pre-incubated with different antibodies and used for adhesion assays to wt ASML matrix. For laminin blocking, the coated matrix was pre-incubated with anti laminin polyclonal serum instead.

2.3.6 CD44vk.d. cells lack a secreted 180 kDa protein

To identify components present in the supernatant that might be responsible for the matrix defect, silver staining of concentrated conditioned supernatant from wt and k.d. cells was performed and revealed a 180 kDa protein, which was greatly reduced in all three k.d. clones (Fig. 22A). The protein was subjected to mass spectrometric analysis and was identified as 'complement component 3' (C3), an element of the innate immune system. The mass spectrometry result was verified using a specific antibody and confirmed, that wt and mock ASML cells secrete complement component 3 and that this is greatly reduced in the k.d. cells. Concentrated conditioned supernatant was subjected to SDS-PAGE under reducing conditions, which leads to separation of C3 into two subunits C3α and C3β (Fig. 22B). However, the restored CD44 expression in the rescue clones failed to restore the secretion of complement component 3, therefore, it can not be ruled out that this is an unspecific off-target effect of the RNAi approach.

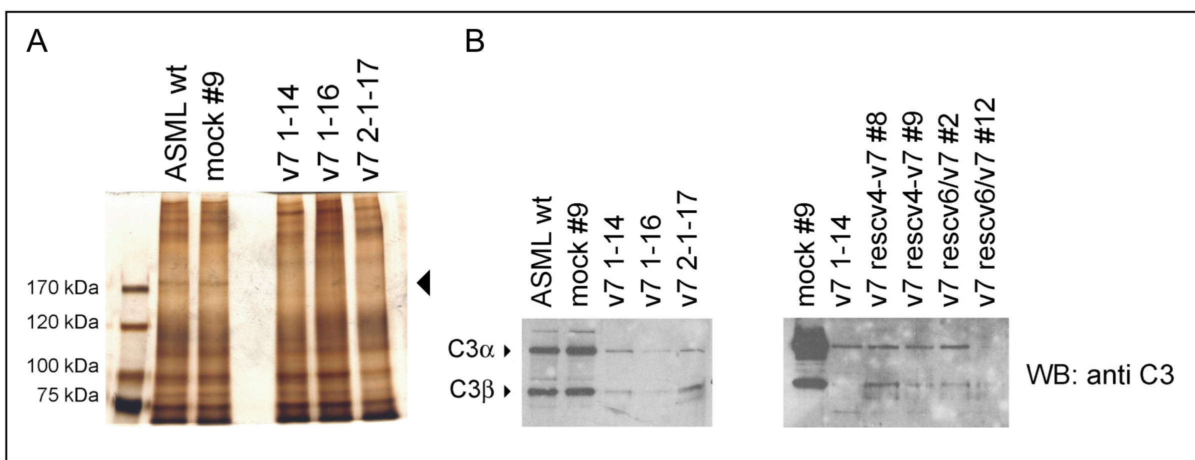

Fig. 22: Differential protein expression of wt and k.d. clones
A. Silver staining of concentrated supernatant of wt and k.d. clones reveals a differentially expressed 180 kDa protein, marked by an arrow. B. Western blot analysis after SDS-PAGE under reducing conditions confirms differential expression of complement component 3, which is not restored in the v71-14 rescue clones.

2.3.7 CD44vk.d. cells exhibit a reduced resistance to apoptotic triggers

Apoptosis resistance is a hallmark of metastatic tumour cells and ASML cells are highly resistant to induction of apoptosis (Matzku et al., 1985). In order to compare susceptibility to apoptosis of wt and CD44vk.d. cells, resistance to the chemotherapeutic drug cisplatin and to γ-irradiation was evaluated. Cells were treated with different concentrations of cisplatin for three days and survival was monitored by MTT staining. For γ-irradiation adherent cells were irradiated with different doses. ASML cells display high drug and radiation resistance and mock transfectants showed comparable levels, while all three CD44vk.d. clones displayed significantly higher susceptibility to both kinds of apoptotic triggers (Fig. 23). The IC_{50} for cisplatin was about 45µg/ml for wt and mock and about 5-10µg for all three CD44v k.d clones. For γ-irradiation the IC_{50} for the k.d. clones was at 250 Gy, while 600Gy killed only about 40% of wt and mock cells. The restored CD44 expression in the rescue clones was not able to reestablish apoptosis resistance (Fig. 24).

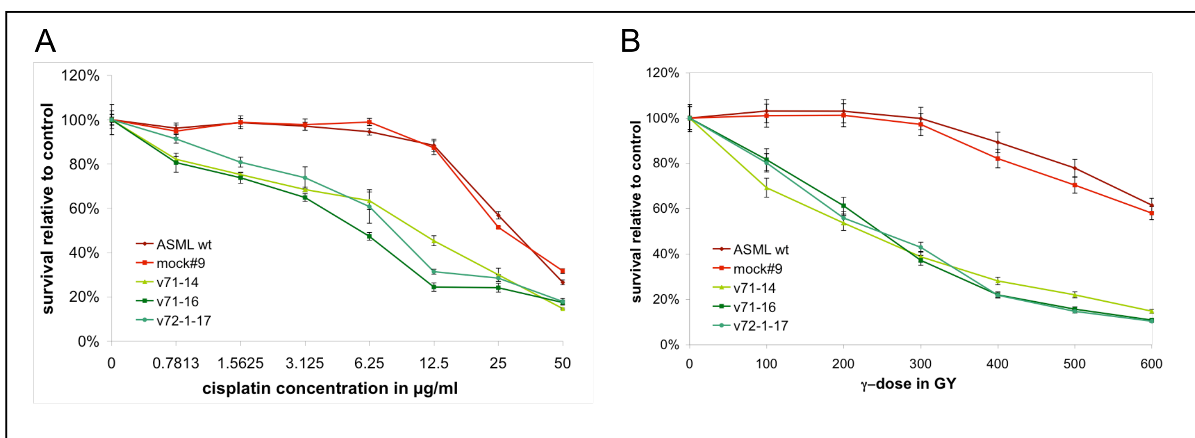

Fig. 23: CD44vk.d. clones are more susceptible to apoptotic triggers
Wt ASML and k.d. clones were treated with different concentrations of cisplatin (A) or subjected to different doses of γ-irradiation (B). Survival was monitored after three days by MTT staining.

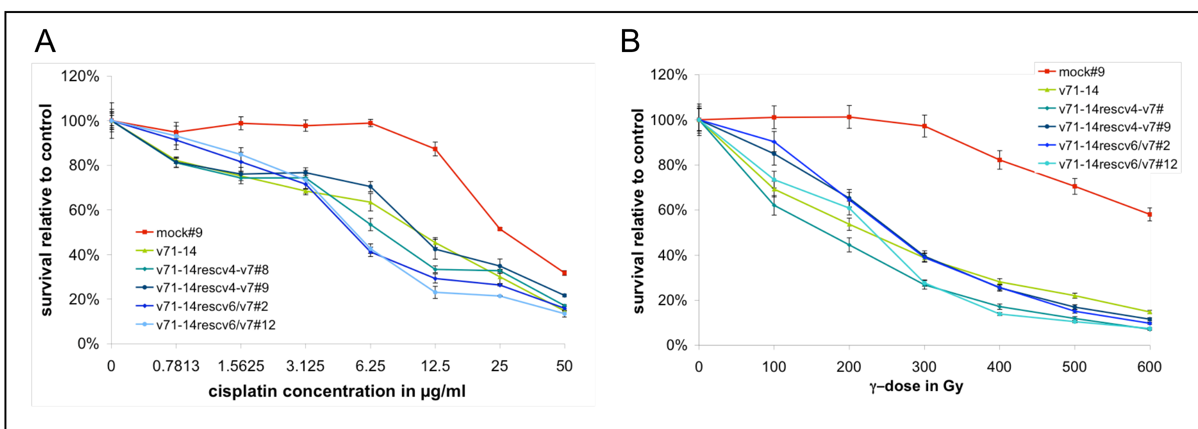

Fig. 24: Restoring CD44 expression does not rescue the apoptosis resistance of CD44vk.d. clones
Mock, k.d. and rescue clones were treated with different concentrations of cisplatin (A) or subjected to different doses of γ-irradiation B). Survival was monitored after three days by MTT staining.

2.3.7.1 Apoptosis resistance is increased by elongated pre-cultivation prior to irradiation

In order to test an influence of the matrix produced by ASML cells on apoptosis resistance, cells were seeded and pre-incubated for either 15 or 48 h before irradiation. The longer cultivation clearly leads to an enhanced resistance. Although the k.d. cells also display higher resistance, they do not reach the level of the wt cells (Fig. 25).

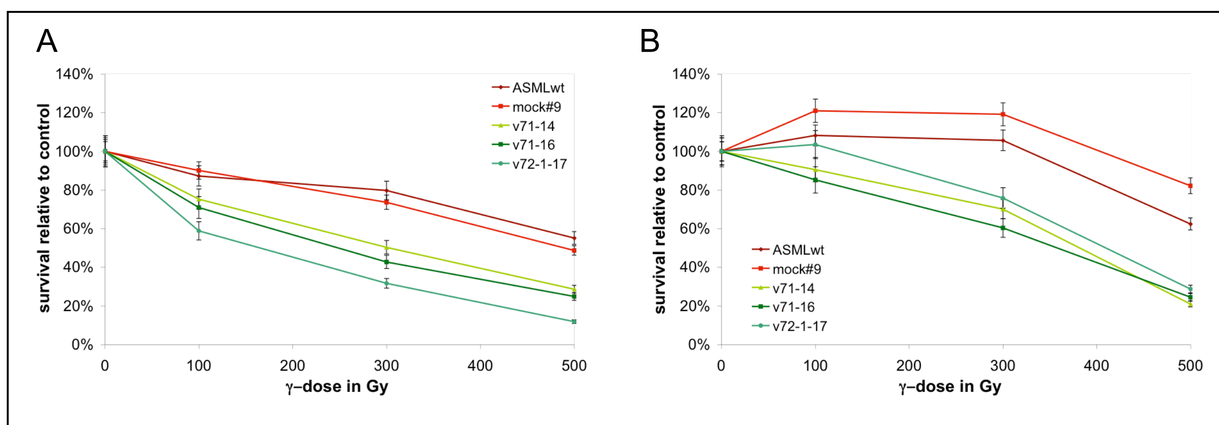

Fig. 25: Influence of cultivation period on resistance to radiation
Cells were cultivated for 15 (A) or 48 hours (B) prior to γ-irradiation. Survival was monitored after three days by MTT staining.

Higher susceptibility to apoptosis of CD44vk.d. cells is not reversible by wt matrix

To test a contribution of the produced matrix to apoptosis resistance, wt, k.d. and rescue cells were seeded onto wt matrix, which had been prepared by EDTA removal of wt cells. Only the wt cells showed a slight increase in resistance, while neither the k.d. clones nor the rescue clones were able to make use of the wt matrix in terms of enhanced apoptosis resistance (Fig. 26).

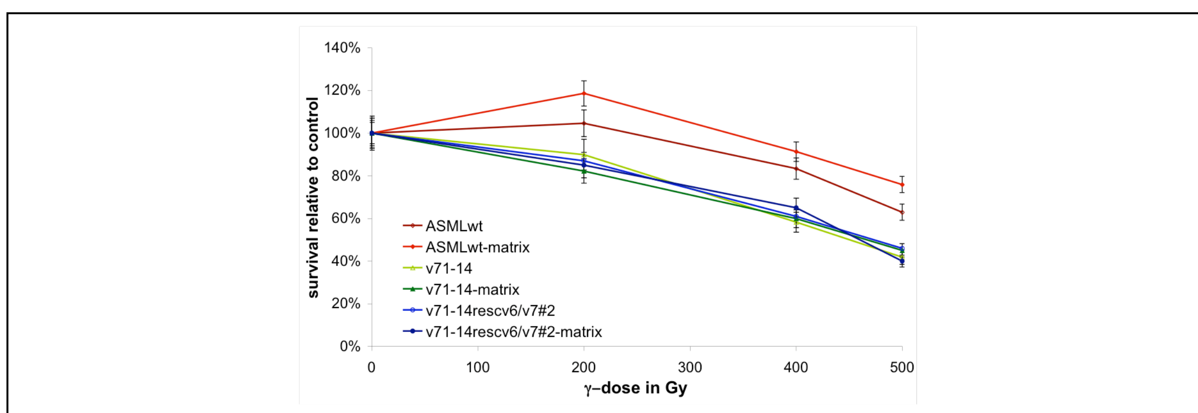

Fig. 26: Wt matrix does not rescue apoptosis resistance of CD44vk.d. cells
ASMLwt, k.d. and rescue clones were seeded on wt matrix and subjected to different doses of γ-irradiation. Survival was monitored after three days by MTT staining.

2.3.7.2 PI3K-Akt, rather than MAPK signalling is involved in apoptosis resistance of ASML cells

The two main pathways influencing apoptosis resistance described to be influenced by CD44 are the MAPK pathway and the PI3K-Akt pathway. Therefore, the influence of specific inhibitors to these pathways was tested for γ-irradiation-induced apoptosis. ASMLwt and CD44vk.d. cells revealed a marked increase in susceptibility to γ-irradiation when treated with the PI3K specific inhibitor LY294002, while an inhibitor of the MAPK pathway (MEK 1/2 inhibitor) had no influence at the applied dose (Fig. 27A, Fig. 28A).

PI3k-Akt signalling is impaired in CD44vk.d. cells

PI3K and Akt inhibitors are known to induce apoptosis in some cancer cells on their own, this was also apparent in the LY294002 treated, but not irradiated controls (Fig. 28A). Therefore, ASMLwt and CD44vk.d. cells were tested for their tolerance for these inhibitors at high concentrations. Using the same survival assay as for cisplatin treatment, adherent cells were treated with different concentrations of either LY294002 or Akt II inhibitor and tested for survival after three days. Wt and k.d. cells display high tolerance for both inhibitors. However, the IC_{50} for wt and mock cells is 125μM for the PI3K inhibitor and 40μM for the AKT II inhibitor, while k.d. cells show the same degree of apoptosis induction already at 50μM and 15-20μM respectively (Fig. 28B+C). This clearly demonstrates an impaired PI3K-Akt signalling in the CD44vk.d. clones compared to wt cells. However, the rescue clones displayed the same reduced tolerance as the k.d. clones. One rescue clone is shown as representative example. The MEK1/2 inhibitor did not induce apoptosis even at very high doses in none of the clones, which excludes a CD44 mediated involvement in MAPK signalling (Fig. 27B).

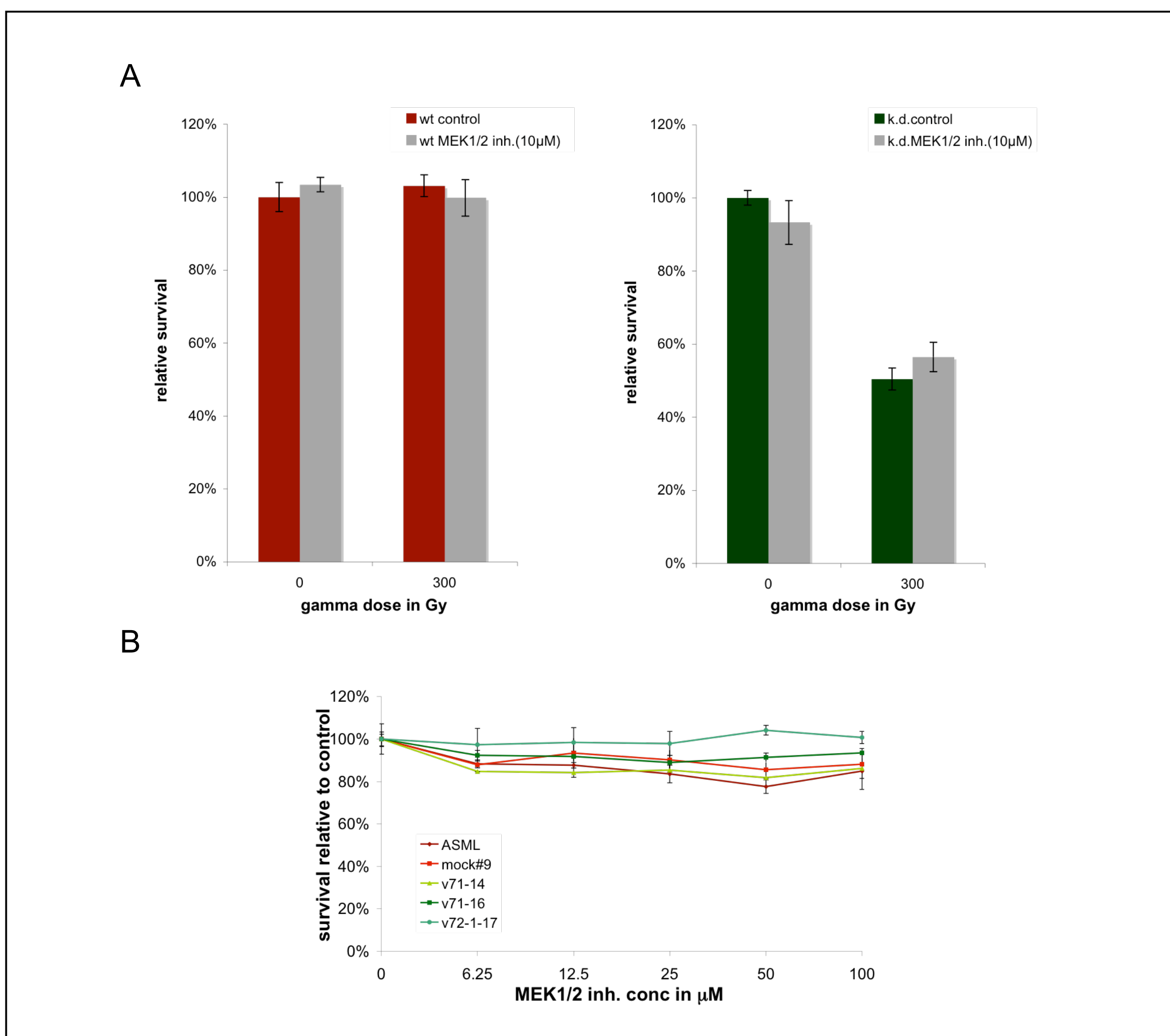

Fig. 27: Interfering with MAPK signalling has no influence on apoptosis resistance of ASML cells

A. ASMLwt (left panel) and CD44vk.d. cells v72-1-17 (right panel) were treated with a MEK1/2 inhibitor at 10µM and irradiated with 300 Gy. Survival was monitored after 3 days by MTT staining. B. ASMLwt, mock and CD44vk.d. cells were treated with different concentrations of the MEK1/2 inhibitor. Survival was monitored after three days by MTT staining.

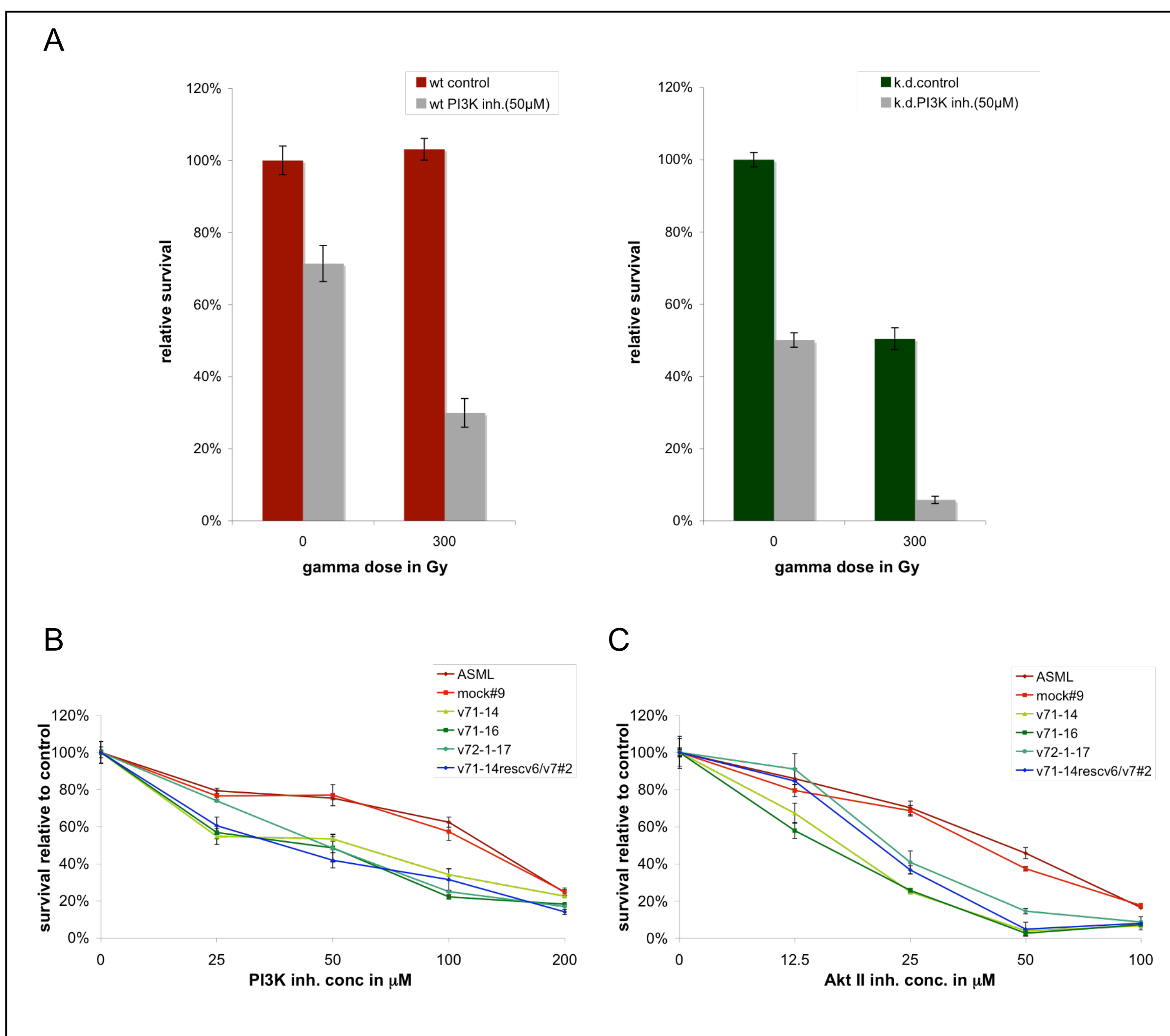

Fig. 28: Interfering with the PI3k-Akt pathway leads to decreased apoptosis resistance and PI3kinase-Akt signalling is impaired in CD44vk.d. cells
A. ASMLwt and CD44vk.d. cells (v71-14) were treated with the PI3K specific inhibitor LY294002 at 50µM and irradiated with 300 Gy. Survival was monitored after 3 days by MTT staining. B. + C. ASMLwt, mock, CD44vk.d. and rescue cells were treated with different concentrations of LY294002 (B) or an Akt II specific inhibitor (C). Survival was monitored after three days by MTT staining.

In order to identify differences in downstream signalling between wt and CD44vk.d. cells, several anti- and proapoptotic molecules were evaluated. Upon cisplatin treatment with 10µg/ml for 24h, cells were lysed, subjected to SDS-PAGE, blotted and tested for expression levels. Phosphorylation of Akt is lowered in the k.d. cells upon drug treatment, while levels stay unaltered in the wt and mock cells. The same was observed for the antiapoptotic molecule Bcl-2, which is only down-regulated in the CD44vk.d. clones after treatment. In correlation with the inhibitor data, phosphoERK levels stay unchanged upon cisplatin treatment in all clones (Fig. 29A+B).

CD44v are able to trigger activation of Akt

Restoring CD44v expression in the rescue clones did not compensate for the impaired PI3K-Akt signalling of CD44vk.d. cells. Therefore, the influence of CD44v on the activation of this pathway was tested by crosslinking surface CD44v on ASMLwt cells. Cells were seeded on plates, coated with anti CD44v6 (A2.6) or BSA as control and lysed after 2h of incubation. After SDS-PAGE and western blotting, phosphorylation of Akt was evaluated. An increase upon crosslinking of CD44v was observed (Fig. 29C). This demonstrates that CD44v can trigger activation of Akt in ASML cells and promote survival in this way.

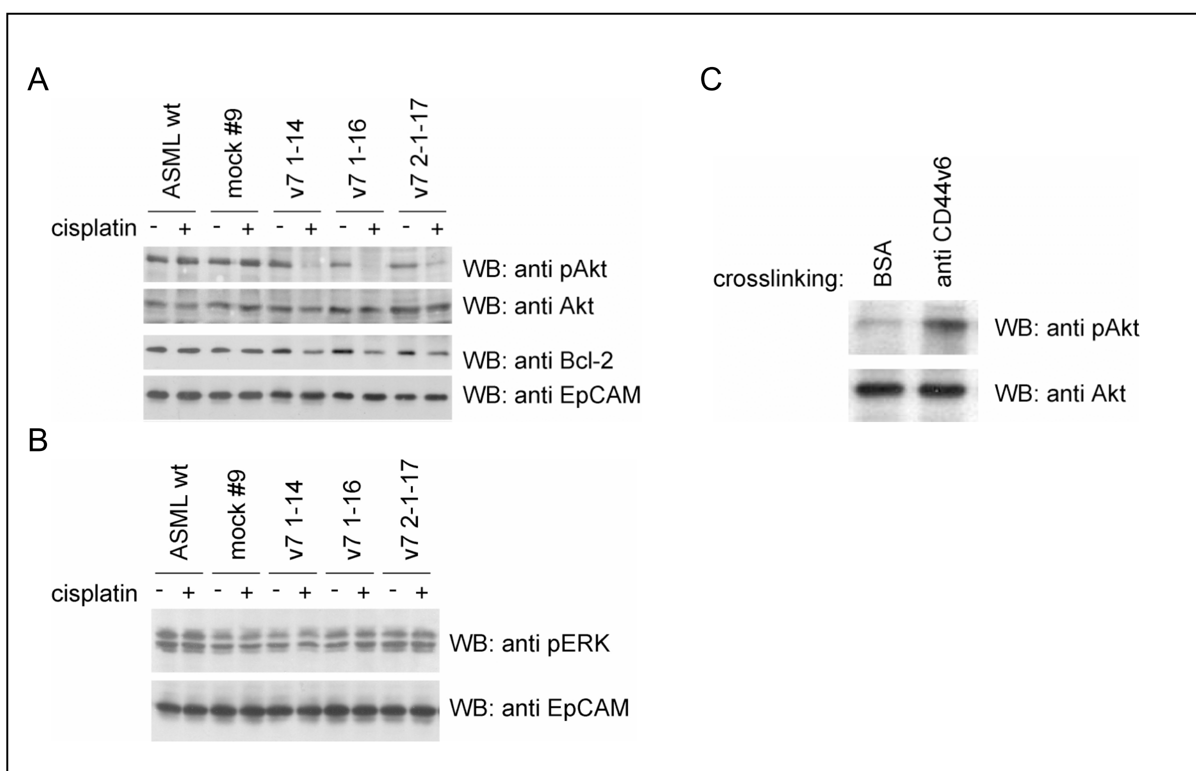

Fig. 29: Reduced survival signalling in CD44vk.d. cells and activation of Akt by crosslinking CD44v

A.+ B. Western blot analysis for untreated and cisplatin (10mg/ml) treated wt and k.d. cells. Cells were lysed 24h after treatment. A. Akt becomes dephosphorylated in the CD44vk.d., but not in the wt cells after drug treatment. Total Akt is used as loading control. Bcl-2 becomes down-regulated only in the CD44vk.d. clones, but remains unaltered in ASMLwt and mock cells upon cisplatin treatment. EpCAM (D5.7) is used as loading control. B. pERK staining does not change upon drug treatment. EpCAM staining (D5.7) is used as loading control. C. Phosphorylation of Akt can be induced by CD44v crosslinking. ASMLwt cells were seeded on plates coated with anti CD44v6 (A2.6) or BSA as control and lysed after 2h. Total Akt is used as control.

3. Discussion

The formation of metastases is the final stage of tumour progression and treatment is still inefficient. Only very recently new therapeutic approaches gained access into clinical application. For example monoclonal antibody therapies, such as 'Herceptin' targeting the HER2 receptor in order to prevent breast cancer metastasis. The basis of these new strategies is a molecular understanding of the mechanisms underlying the process of tumour progression. However, many aspects of metastasis formation remain poorly understood.

The involvement of CD44 in tumour progression and particularly in the development of metastases has been studied for many years, and multiple functions for CD44 and its variant isoforms have been identified (Naor et al., 1997; Marhaba and Zoller, 2004). In this work the role of variant CD44 in different aspects of the metastatic process was investigated in ASML cells, a highly metastatic pancreatic adenocarcinoma. The pSuper RNAi system was used to create stable and variant specific CD44 k.d. cells, which were characterized for their metastatic capacities *in vivo* and *in vitro*. The contribution of CD44v as a cell-cell and cell-matrix adhesion molecule was studied during the multistep process of metastasis formation with special emphasis on interactions with the surrounding. In this respect, several CD44v-mediated features supporting the settlement and survival of tumour cells during lymphatic spread were identified. This highlights the role of CD44 as a multi functional player during the course of metastasis formation through interactions with neighbouring cells and the microenvironment, but also by actively organizing the ECM and finally functioning as signalling molecule, supporting cell survival as well.

Loss of CD44 by stable and variant specific knock-down results in reduced metastatic capacity of ASML cells

The BSp73 cell system comprises of two sublines of the same primary tumour, which display different metastatatic potential. The two most abundant CD44 variants expressed by the highly metastatic ASML cells are v4-v7 and v6/v7, and these isoforms were demonstrated to confer metastatic capacity to otherwise only locally growing AS cells. In order to confirm an essential contribution of these isoforms on the metastatic growth of ASML cells, RNAi constructs were designed to target both isoforms. Two out of three constructs proved to be efficient in down-regulating CD44v expression on ASML cells. Selection and recloning yielded stable clones displaying only hardly detectable residual CD44v expression. Three

clones were established with ‘pSuper-v7’ (subsequently denoted ‘knock-down’ or ‘k.d.’ cells), while no stable clones could be raised with the ‘pSuper-v6/v7’ construct, which would have allowed to easily control construct-specific off-target effects. To confirm any phenotype, arising in the knock-down cells, clones with restored expression of one of the dominant CD44v isoforms were successfully established from one of the k.d. clones (denoted rescue cells). However, the expression level of the CD44v4-v7 rescue did not achieve the wt level.

Upon intra-footpad application, ASML cells spread exclusively through the lymphatics. They display only little local tumour growth, but readily settle in the lymph nodes and the lung, where they form miliary metastastic lesions (Matzku et al., 1983). All three CD44v knock-down clones displayed reduced metastatic growth *in vivo* with severely impaired settlement in the lung. For unknown reasons the injected CD44v4-v7 rescue clone did not grow *in vivo*, probably due to a defect, acquired during *in vitro* culture, which might have allowed the immune system to eliminate the tumour cells. However, five out of six animals, injected with the CD44v6/v7 rescue clone displayed enhanced metastasis formation. Especially when compared to the parental k.d. clone ‘v71-14’, this clearly demonstrates a restored metastatic activity, although the massive tumour burden of the wt was not achieved. The knock-down and the rescued CD44v expression remained stable throughout the *in vivo* experiment, as confirmed by western blotting on re-cultivated tumour cells and by immunohistology on lung sections. In summary, stable and variant-specific loss of CD44 expression in ASML does interfere with the lymphatic spread and lung settlement of these cells. However, more rescue clones will be required to ensure statistical significance of the restored metastatic capacity. It has to be mentioned in this respect, that complement component 3 (C3) was found to be differentially expressed between ASMLwt and CD44vk.d. cells, but could not be restored in the rescue clones, which might argue for an unspecific off-target effect. As C3 is an important molecule of the innate immune system, it cannot be ruled out that this might have affected the *in vivo* experiment. On the other hand, this does not seem very likely, taken into account that the described rescue clone did restore the metastatic capacity of the k.d., irrespective of the unrestored C3 expression.

CD44v knock-down cells display no phenotypic differences or altered growth characteristics

In order to identify defects, which could be causally related to the reduced metastatic growth of ASML CD44vk.d. cells *in vivo*, the cells were studied *in vitro*. The impaired *in vivo* growth is not due to a generally reduced proliferation rate, which was demonstrated by

proliferation assays under high and low serum conditions, nor to a reduced anchorage-independent growth, as shown in soft agar assays. Both, ASMLwt and CD44vk.d. cells are not significantly affected by low serum conditions, as the proliferation rate was only very slightly reduced. Both revealed a very high colony-forming efficacy of 90-95% in soft agar, demonstrating high anchorage-independence. Another way, how CD44 could promote metastasis formation is by up-regulating matrix degrading enzymes to promote invasion. MMP2 and MMP9 are capable of degrading collagen IV, the major collagen of basement membranes, and several studies link hyaluronic acid (HA) and CD44 to MMP2 and MMP9 secretion or activation (Zhang et al., 2002; Isnard et al., 2003; Murray et al., 2004). CD44 can recruit MMP9 to the cell surface, leading to collagen IV degradation and invasion (Yu et al., 1999). Gelatine zymography demonstrated low amounts of secreted MMP2 and MMP9 by ASML, as enzymatic activity was only detectable after concentration of the supernatant. This is in line with the relatively low 'aggressive' growth of ASML cells *in vivo*, which rather grow by displacement than by destruction of host tissue (Matzku et al., 1983, 1985; Raz et al., 1986). No significant differences in MMP2 and MMP9 secretion could be observed between wt and CD44vk.d. cells. It can't be ruled out though, that other MMPs are deregulated in the k.d. cells, which might favour invasive growth. However, the lymphatic spread of tumour cells might require different features and degrading basement membranes might be less mandatory for entrance to the lymphatic vasculature than into blood vessels. The lymphatic spread of tumour cells is still incompletely understood, but an interesting aspect is the involvement of members of the VEGF growth factor family and chemokines in this process. Tumour cells express chemokine receptors on their surface, enabling them to migrate in a chemotactic manner to lymphatic vessels expressing the appropriate ligands. For example, the chemokine receptors CXCR4 and CCR7 are involved in metastasis formation of breast cancer (Muller et al., 2001) and melanoma cells (Wiley et al., 2001). A possible role for chemokine receptors in the lymphatic spread of ASML cells was tested by RT-PCR for CXCR4 and CCR7 expression. However, neither ASML nor AS cells express CXCR4 or CCR7 (data not shown). Therefore, an implication of chemokine receptor expression on the metastatic spread of ASML cells seems rather unlikely.

Agglomeration in HA-rich medium is abrogated in CD44vk.d. cells

ASML cells metastasize via the lymphatics to the lung. As the proliferative capacities of CD44vk.d. cells are unaltered *in vitro*, the stromal surrounding of the lymph nodes or the lung might promote tumour growth. When potential influences of stromal cell lines on

ASML cells were evaluated, it was noted, that conditioned cell culture supernatant of lymph node stromal cells or of lung fibroblasts was able to induce agglomeration of wt, but not of k.d. cells. This feature was completely rescued by restoring CD44v expression. Cell clumping was due to HA-mediated cross-bridging of CD44 molecules on the cells, because it could be induced by high concentrations of HA and was abrogated by hyaluronidase treatment. CD44vk.d. cells are therefore unable to aggregate in stromal supernatant due to the lack of receptor expression. CD44-mediated cell aggregation has been shown to enhance lung metastasis after i.v. injection (Birch et al., 1991, Weber at al., 1996). Thus, it is interesting to note that stromal cells, present at places of major tumour growth of ASML, are capable of producing HA in amounts suitable to induce cell clumping, which could lead to reduced motility and enhanced settlement and thereby promote tumour cell growth. Tumour cell agglomeration has been described to facilitate settlement of tumour cells (Mansury et al., 2002; Glinsky et al., 2003) and the lack of cell aggregation of CD44vk.d. cells may account for reduced metastatic growth.

Impaired matrix production by CD44vk.d. cells

ASML cells adhere very slowly to plastic or matrix components and do not spread, irrespective of the substrate (BenZe'ev et al., 1986). Instead, they display a rounded cell shape, attach very tightly during cultivation and can only be detached by harsh trypsin treatment or very long EDTA exposure. This attachment was reduced in the k.d. cells. When conditioned supernatant of wt and k.d. cells was coated to plastic, both, wt and k.d. cells adhered rapidly to the wt and mock matrix, while the k.d. matrix was clearly less adhesive. Thus it seems likely, that matrixproduction is impaired in the CD44vk.d. cells. This was true for matrix components secreted into the supernatant and for matrix deposited onto the plastic. The rescue clones showed a partially restored matrix production, but could not fully reproduce the adhesive properties of the wt matrix. The components of the matrix are secreted, which could be demonstrated by ultracentrifugation. Hyaluronic acid is most likely needed for proper assembly of the matrix, as hyaluronidase treatment prior to coating disrupts the adhesive properties, but treatment after coating had no influence. This indicates that adhesion of ASML cells is not mediated through CD44-HA interactions, which is confirmed by the finding that CD44vk.d. clones do not display impaired adhesion to the wt-matrix. Instead, collagenase treatment disrupts the adhesive properties of the matrix in a concentration dependent manner, indicating that collagens function as ligands for adhesion. When cells were removed by trypsin or EDTA treatment and the deposited matrix was used

for adhesion, only EDTA detachment of the cells left the matrix intact, while trypsin treatment destroyed the adhesive properties. Adhesion could also be blocked by addition of EDTA during the adhesion assay (data not shown), arguing for Ca^{2+}-dependent adhesion. Antibody blockade demonstrated that β1 integrins are involved, as pre-incubating the cells with anti β1 antibody completely blocked adhesion in a concentration dependent manner. ASML cells express different α chains complementing to β1 at high levels, but anti α2 and anti α3 were not inhibiting adhesion. Anti α6β1 could not be used for antibody blocking, as it induced cell clumping and hindered adhesion in this way (data not shown). Integrins usually do not show high ligand specificity, but α2β1 and α3β1 are known to bind collagen and other matrix components, while α6β1 shows a higher affinity to laminin. However, additional α subunits that were not tested, such as α10β1 and α11β1 were shown to bind collagens as well and might be involved in matrix binding of ASML cells.

Size chromatography under neutral conditions revealed, that the adhesion-mediating components of the matrix are between 600 and 4000kDa in size. This corresponds to a collagen ligand, when organized as multimer or with other matrix components, or present within a HA-lattice. Altered matrix assembly in the k.d. cells may result in a differential distribution of matrix components after size fractionation. The fractions were analysed by western blotting revealing laminin as a component of the adhesive fractions. However, the distribution did not differ in the k.d. matrix. It is possible though, that the resolution of the applied chromatography was not sufficient for detecting differences in size distribution, or that only the order of matrix components is affected, which does not necessarily alter the size of matrix aggregates. However, antibody blocking with a polyclonal serum against laminin did not affect adhesion. Therefore, laminin does not seem to mediate the rapid adhesion, but is present in the matrix. Shed CD44 is not present within the adhesion-promoting fractions, which excludes a mechanism of shedding CD44 to release assembled matrix components. The specific collagen involved in adhesion could not be identified, as the available antibodies did not function in western blotting. Tumour-stroma interactions are important for the pathogenesis of pancreatic cancer, although the matrix production is usually attributed to fibroblastic deposition of ECM (Gress et al., 1995), pancreatic cancer cells have also been described to produce matrix components including collagens (Lohr et al., 1994). CD44 is known to be important for pericellular matrix assembly in chondrocytes, where it functions as a HA-anchor (Knudson et al., 2003; Jiang et al., 2002). Particle exclusion assays with erythrocytes did not reveal any pericellular accumulation of matrix material by ASML cells

grown *in vitro* (data not shown). It might be possible however, that these cells are capable to form a pericellular matrix, when aggregating proteoglycans are added in addition, as shown for other cancer cells (Knudson and Knudson, 1991). The matrix of ASML cells is also deposited on the culture dish and was even more adhesive when prepared in this way than the coated supernatant. Whether the matrix material is actively deposited on the culture dish or just passively bound to the plastic remains open. In conclusion, it seems that cell surface CD44v is needed for proper assembly of matrix components in ASML cells, which most likely is mediated through formation of a HA scaffold. In addition to a HA-anchoring function of CD44, collagens might integrate into the matrix directly via binding to CD44, as CD44-mediated adhesion to collagen containing matrices was described before (Knutson et al., 1996). In chondrocytes, interfering with CD44 cell-matrix interactions results in a 'matrix remodelling' response, which leads to chondrolysis via up-regulation of proteases and enhanced biosynthesis of proteoglycans and HA (Knudson et al., 2000). In this view, it is feasible, that also in ASML cells, CD44 interaction with matrix HA is not only important for assembly, but might also affect matrix biosynthesis.

The ability of ASML cells to secrete their own adhesive matrix could promote metastatic growth at a secondary site in two ways. First, adhesion is supported, enhancing settlement of tumour cells in the lymph nodes and the lung, and second, the matrix could support growth or survival. As metastases have to adapt to the new surrounding at a secondary site, establishing their own matrix should be supportive. Metastasis formation of CD44vk.d. cells may thus be reduced through an impaired ability to generate an adhesive matrix.

Impaired apoptosis resistance of CD44vk.d. cells

Pancreatic adenocarcinomas are aggressive cancers, characterized by invasiveness, rapid progression and high resistance to chemo- and radiation therapy (reviewed by Bardeesy and DePinho, 2002). Similarly, ASML cells exhibit low susceptibility to apoptotic triggers. CD44 is described to be involved in apoptosis resistance and some reports link this feature to variant isoform expression (Bates et al., 1998; Wittig et al., 2000; Marhaba et al., 2003). Metastasis formation and resistance to apoptosis is closely related to the ECM and the microenvironment, known to trigger survival signals. Susceptibility to apoptotic triggers was greatly enhanced in ASML CD44vk.d. cells, using the chemotherapeutic drug cisplatin and γ-irradiation. Because CD44-HA interaction has been described to be involved in apoptosis resistance (Toole, 2004) an impact of the secreted ASML-matrix was tested by enabling

matrix formation for different periods of time prior to irradiation. Indeed, prolonged cultivation increased the apoptosis resistance of both, wt and k.d. cells significantly. Moreover, wt cells could gain further protection by seeding on a preformed matrix, confirming survival supporting functions of the matrix. However, CD44vk.d. cells did not display enhanced resistance on a preformed wt-matrix, arguing for a direct influence of CD44 on survival signalling upon ECM mediated triggers. Yet, the rescue cells were not able to compensate for this, which could have several reasons that will be discussed later.

Cells often react to the substrate by changing the expression of adhesion molecules and alteration of the expressed integrin repertoire may contribute to tumour progression and metastasis formation (Schwartz et al., 1993, Maschler et al., 2005). In pancreatic cancer for example, α6β1 was described to influence metastatic behaviour (Vogelmann et al., 1999). Integrin signalling is also known to affect survival (Lewis et al., 2002). For example, β1 integrin binding to ECM components promotes survival by activating PI3K in small-cell lung cancer cells (Hodkinson et al., 2006). Islets of Langerhans cells are protected from anoikis through β1 ligation with antibodies or by cultivation on collagen IV, which is accompanied by an increase of Akt phosphorylation (Pinkse et al., 2006). However, evaluation of expression levels of α6β1, α6β4 and the subunits α2, α3 and β1 in ASML cells revealed no significant differences, neither between wt and k.d. cells nor upon prolonged cultivation (data not shown). However, not all integrin dimers could be tested due to a lack of suitable antibodies. Besides integrins, also other molecules involved in cell-matrix interactions and contributing to survival could be affected by the matrix defect.

Irrespective of the protective feature of the produced matrix, CD44 is described to interfere with apoptosis in different ways. As ASML CD44vk.d. cells display a reduced resistance to drug treatment and irradiation, it seems unlikely that receptor-mediated apoptosis is the reason. An involvement of multidrugresistance (MDR) cannot account for the observed resistance to radiation as well. Nevertheless, CD44 was shown to influence the expression of MDR genes (Misra et al., 2005; Tsujimura et al., 2006), prompting the analysis of MDR genes in ASML cells. MRP2 and MRP5 are described to be involved in cisplatin transport (Suzuki et al., 2001; Nomura et al., 2005; Oguri et al., 2000), but ASML cells were tested negative for both transporters (data not shown). Instead, CD44v may promote resistance to apoptosis by inducing survival signals. CD44 can signal through the PI3K-Akt and the MAPK pathway to support survival. For example, CD44v6 crosslinking was shown to protect from apoptosis in a thymoma cell line and this was accompanied by persisting

activation of MAPK signalling (Marhaba et al., 2005). In addition, in ASML cells CD44v6 is necessary for c-Met activation by mediating ligand binding through complex formation, resulting in phosphorylation of ERK (Orian-Rousseau et al., 2002). However, activation of MAPK-ERK signalling upon apoptotic triggers was not observed in ASML cells and does not seem to be influenced by CD44v expression, as wt and k.d. cells display comparable pERK levels. In addition, a MEK1/2 specific inhibitor failed to interfere with apoptosis resistance even at concentrations above MEK1/2 specificity inhibiting other members of the MAPK pathway, like ERK1 and MKK3/p38 as well. Instead, inhibition of PI3K enhanced susceptibility to apoptosis in wt and k.d. cells significantly. As inhibitors of PI3K-Akt are known to induce apoptosis at high concentrations on their own, the resistance to this treatment was tested without any additional apoptotic trigger. Indeed, CD44vk.d. cells displayed reduced tolerance to high doses of PI3K and Akt inhibition compared to wt cells, demonstrating impaired PI3K-Akt signalling in CD44vk.d. cells. Accordingly, phosphorylation of Akt is reduced in the k.d. cells but not in wt cells upon ciplatin treatment and the anti-apoptotic molecule Bcl-2 is down-regulated only in the k.d. cells as well. In addition, antibody-crosslinking of surface CD44v induced phosphorylation of Akt in ASML cells. These findings clearly demonstrate that in CD44vk.d. cells one of the major survival pathways is impaired, and that PI3K-Akt signalling contributes, probably in conjunction with other triggers, to the high apoptosis resistance observed in ASMLwt cells. Although the rescue clones could not reverse this defect, an involvement of CD44 was confirmed by CD44v-mediated activation of survival signals through crosslinking.

Activation of PI3K leads to phosphorylation and activation of Akt. Active Akt interferes with the apoptotic machinery by phosphorylating and inactivating the proapoptotic molecule BAD and by inhibiting transcription of pro- and inducing transcription of antiapoptotic molecules. In the active conformation BAD inhibits antiapoptotic members of the Bcl-2 family, which stabilize the mitochondrial membrane and support survival (Igney and Krammer, 2002). Because differences in pAkt levels become apparent after apoptotic triggering, PI3K signalling is not constitutively altered in CD44vk.d. cells, which is observed in many tumour cells through over-expression of PI3K subunits and Akt or lost expression or mutation of the PI3K antagonist PTEN (Igney and Krammer, 2002). Instead, impaired activation of PI3K by CD44 upon apoptotic triggering seems to account for the observed lowered tolerance of CD44vk.d. cells to cisplatin and γ-irradiation. Soluble HA oligomers, competing for HA binding to CD44 can inhibit anchorage-independent growth by suppressing PI3K-Akt (Ghatak et al., 2002). Ligation of CD44 could enhance resistance to

drug treatment by activating the protein tyrosine kinase FAK leading to PI3K association and activation of downstream targets of FAK, such as MAPKs (Fujita et al., 2002). Only interfering with PI3K signalling, but not with the MAPK pathway decreased resistance to apoptosis in this study as well. How CD44v induces PI3K activation remains to be explored in detail, but it seems likely, that CD44v associated protein kinases are involved. However, no associations with FAK or PI3K could be demonstrated by immunoprecipitations in ASML cells (data not shown). Another possibility might be an inhibiting activity of CD44v on PI3K antagonists, as soluble HA oligomers were demonstrated to be capable to stimulate the expression of the PI3K antagonist PTEN (Ghatak et al., 2002). An involvement of the described matrix production in apoptosis resistance seems likely as well. Activation of Akt upon crosslinking of CD44v argues for CD44 acting directly as receptor for matrix components, but, because crosslinking induces clustering as well, CD44v could also cooperate with other molecules, such as integrins to activate survival signalling in ASML cells. As β1 integrin was identified to mediate adhesion of ASML cells and ligation of β1 integrins is known to support survival, both possibilities seem feasible and might even act synergistically.

CD44v-mediated apoptosis resistance and adhesiveness as crucial contributions during the lymphatic spread of ASML cells

The importance of apoptosis resistance for metastatic cancer cells and especially survival induced by the microenvironment is becoming more and more apparent. As reviewed by Mehlen and Puisieux (Mehlen and Piusieux, 2006), one way to explain the very rare incidence of metastatic outgrowth (Liotta et al., 1978; Varani et al., 1980) is to look at apoptosis acting as a multistep barrier to metastasis at three crucial steps. During the initial step of metastasis, cells detach from the underlying ECM and the actin cytoskeleton is disrupted leading to cell rounding. These early events usually induce apoptotic processes called anoikis and amorphosis (Streuli et al., 1999, Martin et al., 2004) and cancer cells must become resistant to these apoptotic stimuli. The second step during which apoptosis can be induced is the process of intravasation and during residence in the circulation. Cell death may be induced by mechanical stress associated with entrance into the blood stream (Weiss et al., 1993; Ziegler et al., 1998) or be mediated by the immune system, known as immune surveillance (Jakobisiak et al., 2003). Indeed, cancer cells display a high frequency of apoptosis when injected into the circulation, while over-expression of antiapoptotic molecules like Bcl-2 can increase the number of metastatic lesions at secondary sites (Wong

et al., 2001). Moreover, the tumour suppressor p53 was shown to facilitate experimental metastasis by promoting the survival of tumour cells in the circulation (Nikiforov et al., 1996). Finally, after settlement cancer cells have to survive at the secondary site to form metastases, the third phase, when cancer cells display high frequency of apoptosis. Enhanced antiapoptotic signalling is supporting metastasis formation at this step as well (Wong et al., 2001; Luzzi et al., 1998).

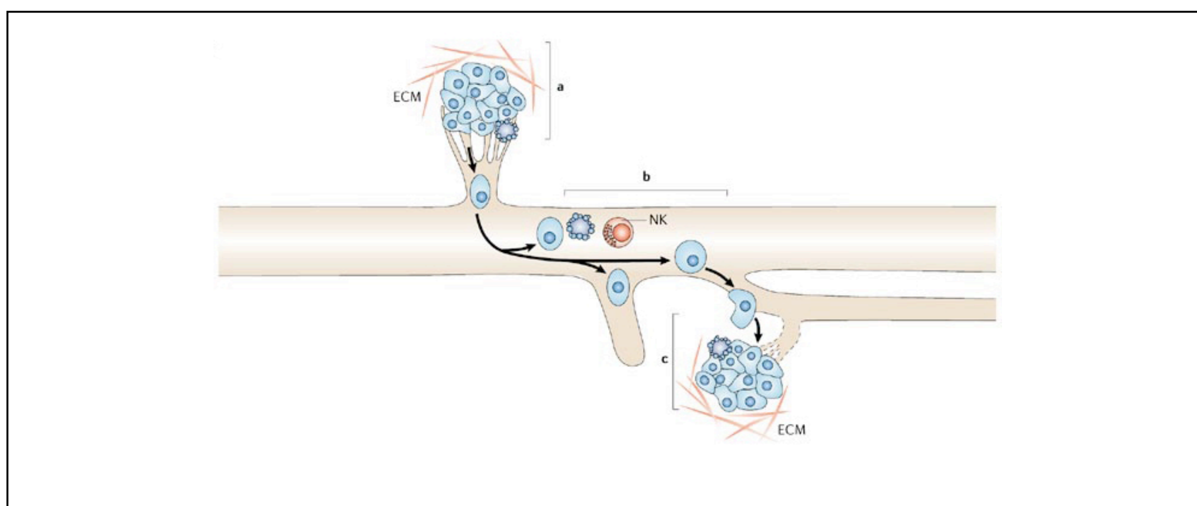

Fig. 30: Apoptosis at three crucial steps during the metastatic spread of tumour cells
a. Detachment from the primary tumour mass induces anoikis. b. Cell death in the circulation through immune surveillance or mechanical stress. c. Apoptosis after extravasation during micrometastasis formation at the secondary site. ECM extracellular matrix, NK natural killer cell. *Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Mehlen and Puisieux., 2006).*

The observed CD44-mediated apoptosis resistance could protect ASML cells at different points in the metastatic process. ASML cells do not form tight cell-cell contacts under *in vitro* culture conditions and display only little local tumour growth *in vivo*. CD44vk.d. cells do not display reduced anchorage-independence. Thus, the initial step of dissemination from the primary tumour is probably not limiting for ASML metastasis formation. ASML cells spread exclusively via the lymphatics, therefore they do not need to invade blood vessels, and because lymphatics lack the tight inter-endothelial junctions of blood vessels (Alitalo and Carmeliet, 2002), intravasation should be less stressful for tumour cells and probably even allow small cell aggregates to enter. The growth in the draining lymph nodes was already reduced in CD44vk.d. cells, which argues for a growth and/or survival advantage in this environment, but the settlement in the lung seems to be most severely impaired in the CD44vk.d. clones. While cells from connective tissue tumours, like fibrosarcomas usually migrate individually, carcinomas were described to often migrate

collectively as small aggregates (Friedl and Wolf, 2003). It seems likely that ASML cells start to migrate as individual cells, enter the lymphatics and aggregate only once they reach the draining lymph node and are confronted with a HA-rich environment. This might be favouring growth and survival within the lymph node and the lymphatic vessels and probably facilitate further travel within the lymphatic system. The physiology of lymph nodes and the relatively low shear flow of lymphatic fluid was discussed in this context to favour the concentration of tumour cell aggregates in the lymph nodes, which then may support the growth of local metastases that could serve as 'bridgeheads' for further dissemination (Sleeman, 2000). In comparison, the metastatic progression in the lung is highly inefficient, which could be explained in this respect by the large capillary bed, leading to dispersal of individual tumour cells (Chambers et al., 2002). In addition, the lung seems to be harder to colonize than the lymph nodes also for ASML cells, as even the rapidly growing 'v71-16' clone exhibited impaired settlement in the lung. This could be due to the lack of CD44v-mediated aggregation or adhesion or again to increased apoptosis. Aggregation may facilitate arrest in the capillary bed, which in turn would enable matrix deposition and subsequent firm adhesion. Adhesion to matrix possibly then promotes survival at the secondary site.

Strong CD44-HA interaction can promote certain steps of the metastatic process, while weak CD44-HA interaction might favour others. For example, release of cells from the primary tumour is accompanied by reduced expression of CD44 in endometrial carcinoma (Fujita et al., 1994). CD44 was shown to mediate attachment of circulating cancer cells to the endothelial vessel wall through interaction with HA (De Grendele et al., 1996/1997). Moreover, survival during micrometastasis formation can be mediated by CD44-HA binding. This was shown in a mammary carcinoma cell line transfected with a soluble form of CD44, which competed for HA-binding. The cells were able to infiltrate lung tissue but underwent apoptosis thereafter and failed to form lung tumours (Yu et al., 1997). In addition, an influence of CD44 glycosylation on HA binding was demonstrated (Skelton et al., 1998; English et al., 1998). Because it is well known that glycosylation patterns are changed in cancers and that changes can increase with tumour progression (Alhadeff, 1989), it seems likely that changes in the HA binding ability of CD44 can by this means support the metastatic process at different steps. The loss of CD44v may therefore not only suppress metastasis formation by the inability to bind HA, but probably also because of the lost ability to modify this interaction in a dynamic manner. This could still be the case in the rescue clones and might be a reason for the limited ability of these cells to restore all observed phenotypes of the k.d. cells. For instance the untranslated regions (UTRs) of the CD44

transcript could be functionally involved, as the rescue cDNAs did not contain the endogenous UTRs. Recently the 3’ UTR of CD44 was described to be involved in translational control by stabilizing the transcript through bound IMPs, a family of ribonucleoproteins (RNPs). In addition, different CD44 variants exhibited differences in their 3’UTR sequence and regulation by IMPs was isoform specific. Moreover, interfering with IMP function led to abrogation of invadopodia formation, which was attributed to the deregulation of CD44 (Vikesaa et al., 2006). IMPs are described to be involved in mRNA localization, with implications on coordinated spatio-temporal protein expression and overexpression of IMPs is implicated in cancer progression (Ioannidis et al, 2001; Tessier at al., 2004). This mechanism provides an additional level of complexity to CD44 regulation and it cannot be ruled out that similar mechanisms are responsible for the failure of the restored CD44 expression to rescue all observed phenotypes. The fact that the rescue clones restored the metastatic ability as well as the matrix production in part, but failed to restore apoptosis resistance is most easily explained by the lower overall CD44 expression level compared to the wt situation. Specifically, the initiation of signal transduction might depend on the expression level. It is also possible, that ASML cells require both isoforms, which were affected by the knock-down or that additional isoforms were targeted, but not restored.

Future perspectives and conclusions

Differential contributions of untranslated sequences during the course of metastasis formation seem very interesting with respect to CD44 regulation. Rescue constructs, carrying endogenous UTRs could be applied to the established k.d. cell system, to study influences on the metastatic growth in detail. As CD44 is reported to take part in the regulation of gene expression, it might be worthy to look for deregulated gene or protein expression in the k.d. cells, for example by microarray and CHIP analysis. With respect to the described matrix production, changes in the expression of matrix components or molecules involved in matrix remodelling, such as proteases could be addressed. In addition, the role of CD44v in matrix organization could be analysed by studying morphological differences by electron or fluorescence microscopy on deposited matrix material.

As the rescue clones did not revert all CD44vk.d. phenotypes, e.g. the reduced expression of ‘complement component 3’, the CD44 specificity of these observations has to be critically judged. The only alternative way to control any phenotype arising by RNAi is to reproduce the same phenotype using a second construct. However, no stable clones could be established with the second functional construct. Due to a low transfection efficiency of

ASML cells, transient transfection is not practical either. However, the use of an inducible RNAi system could solve this problem, as generation of stable clones should be less problematic.

This work demonstrates an essential contribution of CD44v to the metastatic capacity of ASML cells. However, considering the differences of AS and ASML cells, CD44v seems to support metastasis formation in different ways depending on the cellular background. AS cells adhere to and spread on different matrices, while ASML cells hardly adhere to any substrate except their own matrix. Therefore matrix generation might be a crucial feature of ASML cells but not for AS. Abrogation of HA binding had no influence on the metastatic capacity of AS cells transfected with CD44v (Sleeman et al., 1996) and hence, CD44v-mediated aggregation in lymph nodes or the lung might not be limiting for AS cells, as these cells tend to build up tight cell-cell contacts anyhow. Still, the induction of agglomeration might be an essential contribution of CD44v on metastasis formation of ASML cells. In addition, upon transfection with CD44v, AS cells did not gain apoptosis resistance (data not shown). Accordingly, the multitude of differentially expressed genes between AS and ASML seems to influence the way CD44v contributes to metastatic progression of these cells. This strengthens the idea that actions of CD44v depend on the cellular background, which is not surprising given the heterogenous nature of CD44 functions and interactions.

In summary, variant-specific down-regulation of CD44 in a highly metastatic pancreatic adenocarcinoma is accompanied by a markedly reduced metastatic capacity and settlement in the lung. Defects in proliferative or anchorage-independent growth were ruled out. Likewise, a change in the level of MMP2 and MMP9 secretion was not observed, which would have argued for differences in invasive capacities. On the other hand, several differences were examined and characterized, that could account for the observed metastatic defect. First, CD44vk.d. cells lost the ability to aggregate in a stromal surrounding due to their inability to crosslink surface CD44 through hyaluronic acid. Second, ASML cells secrete a highly adhesive matrix, containing HA, collagen and laminin, to which they adhere rapidly via β1 integrins and which might contribute to apoptosis resistance. CD44v are most likely involved in matrix production by assembly of a HA-rich scaffold and therefore CD44vk.d. cells display an impaired matrix generation. Finally, CD44vk.d. cells are clearly less resistant to apoptotic triggers, as demonstrated for drug resistance and γ-irradiation, which seems to be the cause of impaired PI3K-Akt signalling due to the loss of CD44v-mediated activation.

These results support the idea of CD44 as a molecule with multiple features that, due to its compositional and functional heterogeneity influences tumour progression and metastasis formation at several different steps. Even in the studied cell system, which reflects only the advanced steps of tumour progression, CD44v contributes to the process of metastasis formation in several ways: as a cell-cell adhesion molecule, as organizer of extracellular matrix components and as signalling molecule influencing survival. Importantly, all the described mechanisms are based on complex interactions of CD44v on the tumour cell with its surrounding in diverse ways. This underlines the importance of communication between cancer cells and their microenvironment for the metastatic cascade, which is a major subject of recent investigation and possesses growing importance for future therapeutic strategies.

4. Materials and Methods

4.1 Materials

4.1.1 Chemicals

Agar	Fluka, Buchs, Schweiz
Agarose	Sigma, Seelze
3-Amino-9-ethyl-carbazol (AEC)	Sigma, Seelze
Ammoniumpersulfate (APS)	Sigma, Seelze
Ampicillinsulfate	Sigma, Seelze
Biotin-X-NHS	Calbiochem, Darmstadt
Brij98	Fluka, Buchs, Schweiz
Brilliant Blue G-Colloidal Concentrate	Sigma, Seelze
Bromphenolblue	Merck, Darmstadt
Cisplatin (cis-diammineplatimun(II)dichloride)	Sigma, Seelze
Ethidiumbromide	Merck, Darmstadt
Ethylendiamintetraessigsäure (EDTA)	Sigma, Seelze
Fetal calf serum (FCS)	Sigma, Seelze
Formaldehyde 37%	Merck, Darmstadt
Glucose	Merck, Darmstadt
Glutamine	Life Technologies, Karlsruhe
Glycerine	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Yeast extract	Gibco BRL, Eggenstein
HEPES	Sigma, Seelze
Hyaluronic acid (from rooster comb)	Sigma, Seelze
Kanamycinsulfate	Calbiochem, Darmstadt
Lubrol WX (17A17)	Serva, Heidelberg
Milk powder	Roth, Karlsruhe
Mayer's Hämalaun	AppliChem, Darmstadt
β -Mercaptoethanol	Sigma, Seelze
Mowiol (4-88)	Calbiochem, Darmstadt
Natriumorthovanadat	Sigma, Seelze
Paraformaldehyde	Sigma, Seelze
Penicillin	Sigma, Seelze
Pepton 140	Gibco BRL, Eggenstein
Phenylmethylsulfonylfluorid (PMSF)	Sigma, Seelze
Protease Inhibitor Cocktail	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Pharmacia, Freiburg
Rotiphorese Gel 30 (Acrylamid-Mix)	Roth, Karlsruhe
Sepharose CL-6B	Amersham Biosc., Freiburg
Sepharose Superdex 200	Amersham Biosc., Freiburg
Streptomycinsulfate	Sigma, Seelze
TEMED	Sigma, Seelze

^{3}H-Thymidine	Amersham Biosc., Freiburg
Triton X-100	Sigma, Seelze
Trypsin	Sigma, Seelze
Tween 20	Serva, Heidelberg

All other chemicals, not listed were analytical grade and purchased from Sigma (Seelze), Calbiochem (Darmstadt), Serva (Heidelberg) or Applichem (Darmstadt).

4.1.2 Enzymes

resctriction enzymes	MBI Fermentas, St. Leon Rot
Taq-polymerase	MBI Fermentas, St. Leon Rot
PWO-polymerase	Promega, Mannheim
T4-Ligase	Promega, Mannheim
T4-Polynucleotidkinase (T4-PNK)	Promega, Mannheim
Klenow fragment	Promega, Mannheim
ImProm II reverse transcriptase	Promega, Mannheim
calf intestinal alkaline phosphatase (CIAP)	Promega, Mannheim
hyaluronidase type IV-S from bovine testis	Sigma, Seelze
collagenase, type 2	PAA, Coelbe

4.1.3 Chemical inhibitors

LY294002(PI3-K-inhibitor)	Calbiochem, Darmstadt
Akt II inhibitor	Calbiochem, Darmstadt
MEK1/2-inhibitor	Calbiochem, Darmstadt

4.1.4 Nucleotide and protein standards

100bp Gene Ruler	MBI Fermentas, St. Leon Rot
1 kb Gene Ruler	MBI Fermentas, St. Leon Rot
Prestained Protein ladder	MBI Fermentas, St. Leon Rot

4.1.5 Kits

Qiaprep Spin miniprep kit	QIAGEN, Hilden
Qiaquick Gel Extraction Kit	QIAGEN, Hilden
Qiaquick midi prep kit	QIAGEN, Hilden
ECL Western Blotting Detection Reagents	Amersham Biosc., Freiburg
Vectastain ABC kit	Vector Laboratories, Burlingame, USA
TRI Reagent	Sigma, Seelze

4.1.6 Vectors

vector	description	company
pSuper.gfp/neo	eukaryotic expression vector for RNAi	Oligoengine, Seattle, USA
pcDNA3.1(+)Neo	eukaryotic expression vector	Invitrogen, Karlsruhe
pcDNA3.1(+)Hygro	eukaryotic expression vector	Invitrogen, Karlsruhe

4.1.7 Primers and oligos

Oligonucleotides were purchased from Operon Biotechnologies (Koeln)

oligo	sequence
v6RNAi-sense	5'–TCGAGGCACAACAGAAGAAGCAGCTACCCAGAACTTCCTGT CATTCTGGGTAGCTGCTTCTTCTGTTGTGCTTTTT–3'
v6RNAi-antisense	5'–CTAGAAAAAGCACAACAGAAGAAGCAGCTACCCAGAATGA CAGGAAGTTCTGGGTAGCTGCTTCTTCTGTTGTGCC–3'
v7RNAi-sense	5'–TCGAGAGAATGACAACACAGAGTCAAGAGGATGCTTCCTGT CACATCCTCTTGACTCTGTGTTGTCATTCTTTTTT–3'
v7RNAi-antisense	5'–CTAGAAAAAAGAATGACAACACAGAGTCAAGAGGATGTGA CAGGAAGCATCCTCTTGACTCTGTGTTGTCATTCTC–3'
v6/v7RNAi-sense	5'–TCGAGCAACTGCCTCAGCCCACAACTTCAAGAGAGTTGTGG GCTGAGGCAGTTGTTTTT–3'
v6/v7RNAi-antisense	5'–CTAGAAAAACAACTGCCTCAGCCCACAACTCTCTTGAAGTT GTGGGCTGAGGCAGTTGC–3'
T7-BglII-R	5'–GAGCTAGATCTAATACGACTCACTATAGGG–3'
EcoRI-CD44-F	5'–GCAGTGAATTCCCACCATGGACAAGGTTTGGTGGCAC–3'
CD44-XhoI-Stop-R	5'–CGACGCTCGAGGCACTACACCCCAATCTTC–3'
v7resc-F	5'–TACTCAGTCTCAAGAGGATG–3'
v6/v7resc-F	5'-CAACTGCCAGCGCGCACAACAAC–3'
CXCR4-F	5'–CCCTCCTCCTGACTATCCCT–3'
CXCR4-R	5'–TGACTCTGTGGAGACGGAAGA–3'
CCR7-F	5'–GGCGAGAACACCACCGTGGAC–3'
CCR7-R	5'–TTCTGGAGGCCGCTGTAGA–3'
SDF1-F	5'–GCCAAGGTCGTCGCTGTGCT–3'
SDF1-R	5'–TTGGATCCACTTTAATTTCGG–3'

4.1.8 cDNAs and constructs

CD44v4-v7 cDNA and CD44v6/v7 cDNA (Gunthert et al., 1991; Rudy et al., 1993) were used as template for PCR amplification. All constructs were verified by sequencing.

construct	vector	insert	cloning sites
pSuper-v6	pSuper.gfp/neo	CD44v6RNAi-hairpin	BglII-HindIII
pSuper-v7	pSuper.gfp/neo	CD44v7RNAi-hairpin	BglII-HindIII
pSuper-v6/v7	pSuper.gfp/neo	CD44v6/7RNAi-hairpin	BglII-HindIII
meta1-rescv7	pcDNA3.1Hygro	CD44v4-v7-rescuev7-cDNA	EcoRI-XhoI
meta2-rescv7	pcDNA3.1Hygro	CD44v6/v7-rescuev7-cDNA	EcoRI-XhoI
meta1-rescv6/v7	pcDNA3.1Hygro	CD44v4-v7-rescuev6/v7-cDNA	EcoRI-XhoI
meta2-rescv6/v7	pcDNA3.1Hygro	CD44v6/v7-rescuev6/v7-cDNA	EcoRI-XhoI
CD44meta1	pcDNA3.1Hygro	CD44v4-v7-cDNA	EcoRI-XhoI
CD44meta2	pcDNA3.1Hygro	CD44v6/v7-cDNA	EcoRI-XhoI

4.1.9 Antibodies

4.1.9.1 Primary antibodies

antibody (clone)	application	company/reference
mouse anti rat CD44pan (Ox50)	FACS, IP, blocking exp.	Paterson et al. 1987
mouse anti ratCD44v6 (A2.6)	FACS, IP, blocking exp., immunohistology, WB,	Matzku et al. 1989
mouse anti rat EpCAM (D5.7)	FACS, IP, WB	Wurfel et al., 1999
mouse anti rat D6.1A (D6.1)	FACS, IP, WB	Claas et al., 1998
mouse anti rat C4.4A (C4.4)	FACS, immunohistology	Rosel et al., 1998
mouse anti rat transferrin receptor (Ox26)	FACS, IP, WB	European Collection of Animal Cell Culture
mouse anti rat α3β1 integrin (Ralph3.1)	FACS, IP, blocking exp.	Developmental Studies, Hybvridoma Bank
mouse anti rat α6β4 integrin (B5.5)	FACS, blocking exp.	Herlevsen et al., 2003
mouse anti rat β1 integrin (Ha2/5)	FACS, IP, blocking exp.	BD Biosciences, Heidelberg
rabbit anti human α3 integrin (polyclon.#AB1920)	WB	Chemicon, Temecula, Canada
hamster anti rat α2 integrin (Ha1/29)	FACS, blocking exp.	BD Biosciences, Heidelberg
mouse anti rat α6β1 integrin (MAB1410)	FACS	Chemicon, Temecula, Canada
mouse anti rat α4 integrin (MRα4-1)	FACS	BD Biosciences, Heidelberg

hamster anti rat α1 integrin (Ha31/8)	FACS	BD Biosciences, Heidelberg
hamster anti mouse α5 integrin (HMα5-1)	FACS	BD Biosciences, Heidelberg
mouse anti rat β2 integrin (WT.3)	FACS	BD Biosciences, Heidelberg
mouse anti rat β3 integrin (F11)	FACS	BD Biosciences, Heidelberg
mouse anti human ERK (16)	WB	BD Biosciences, Heidelberg
mouse anti human pERK1/2 (20A)	WB	BD Biosciences, Heidelberg
mouse anti human Akt (2)	WB	BD Biosciences, Heidelberg
mouse anti human pAkt (104A282)	WB	BD Biosciences, Heidelberg
mouse anti human Bcl-2(7)	WB	BD Biosciences, Heidelberg
rabbit anti human laminin (polyclon. #600-401-116-05)	WB, blocking exp.	Rockland, Gilbertsville, USA
goat anti rat complement C3 (polyclon.#5571304564)	WB	MP Biomedicals, Aurora, Ohio, USA

4.1.9.2 Secondary antibodies/ reagents

antibody	application	company
anti mouse-IgG-TxRed	immunofluorescence	dianova, Hamburg
anti mouse-IgG-biotin	immunohistology	dianova, Hamburg
anti mouse-IgG-HRP	WB	Rockland, Gilbertsville, USA
anti goat-IgG-HRP	WB	Rockland, Gilbertsville, USA
anti rabbit-IgG-HRP	WB	Rockland, Gilbertsville, USA
Extravidin-Peroxidase	WB	Sigma, Seelze
anti mouse-IgG-PE	FACS	dianova, Hamburg
anti mouse-IgG-APC	FACS	BD Biosciences, Heidelberg
anti mouse-IgG-FITC	FACS	dianova, Hamburg
anti rabbit-IgG-PE	FACS	dianova, Hamburg
anti hamster-IgM-FITC	FACS	dianova, Hamburg

4.1.10 Cell lines

Cell line	source	reference
BSp73ASML	Rattus norvegicus, pancreas adenocarcinoma	Matzku et al., 1983
BSp73AS	Rattus norvegicus, pancreas adenocarcinoma	Matzku et al., 1983
BSp73AS-14	Rattus norvegicus, pancreas adenocarcinoma BSp73AS, transfected with cDNA for CD44v4-v7	Gunthert et al., 1991
HEK 293T	Homo sapiens, renal cell carcinoma	Graham et al., 1977
Fibroblasts	Rattus norvegicus, immortalized lung fibroblasts	Weth, 2000
ST-A4	Rattus norvegicus, immortalized lymph node stromal cell	LeBedis et al., 2002
ST-B12	Rattus norvegicus, immortalized lymph node stromal cell	LeBedis et al., 2002

4.1.11 Animals

BDX rats were bred at the animal facilities of the German Cancer Research Center (DKFZ), kept under pathogen free conditions and fed with sterilized food and water.

4.2 Methods

4.2.1 Molecular biology

4.2.1.1 Bacteria

For all bacterial work DH5α were used. DH5α were cultured in liquid Luria Bertani (LB) medium (10g peptone, 5g yeast extract, 10g NaCl per l) or on plates containing 1% (w/v) bacto agar and either 60μg/ml ampicillin or 50μg/ml kanamycin for selection. Transformations were carried out with an 'EasyJect' electroporator (Eurogentec, Seraing, Belgium) using standard protocols for electro-transformation.

DH5α genotype: Φ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K^-, m_K^+), *sup*E44, *relA*1, *deoR*, Δ(*lacZYA*-*argF*)U169;

4.2.1.2 Plasmid preparation

All plasmid preparations were carried out using either the 'QIAprep spin Miniprep kit' or 'plasmid midi prep kit' (both QIAGEN, Hilden) according to manufacturer's instructions.

4.2.1.3 RNAinterference (RNAi) construct design and cloning

In order to create stable CD44 knock-down clones the pSuper plasmid based RNAi system (oligoengine, Seattle, USA) was used. The pSuper plamids facilitate expression of 'small hairpin RNAs' (shRNAs) under the control of the human RNA polymerase III promoter H1, which were shown to enter the endogenous RNAi pathway and induce degradation of the target mRNA. A GFP reporter and a neomycin resistance are included in the vector for detection and selection of transfected cells.

Fig. 31: pSuper.gfp/neo inserts and predicted haipin structure
A. ds inserts for cloning into pSuper.gfp/neo. The target sequences lie within exon v6, v7 or span the border of exon v6/v7. Due to the self-complementary sequence, transcripts fold into a hairpinstructure, which is processed by the endogenous RNAi machinery to a functional siRNA leading to target mRNA degradation. B. Predicted hairpin structure for construct 'v7'.

The target sites were chosen on the premise to target the two most abundant CD44 variant isoforms, expressed by ASML, which are v4-v7(meta1) and v6/v7(meta2). Target sites for constructs 'v6' and 'v7' were chosen randomly, except that stretches of more than 3 A-residues were avoided. Homologies to other genes were excluded by 'BLAST' search. For

the 'v6/v7' construct, which spans the border of the v6-v7 exons, the RNAi target site validation program 'Sfold' was used, which is provided on the homepage of Wadsworth Center-NYS, Department Of Health (http://sfold.wadsworth.org; Ding et al., 2004; Ding and Lawrence, 2001/2003) and includes several parameters like internal stability of the siRNA-dublex and target site accessibility. The constructs consist of a self complementary sequence stretch comprising the 21-28 bp target sequence and the corresponding reverse complement, separated by a 10 bp loop and followed by a stretch of 5 T-residues leading to termination of transcription. Sense and antisense oligos were designed to elicit sticky ends upon annealing. 200pmol of each ss oligo were diluted in ligation buffer (Promega, Mannheim), boiled for 2min and slowly cooled down to room temperature to ensure proper annealing. Because oligos were initially designed for XhoI-XbaI cloning, the ds inserts were partially filled up and ligated into the, as well partially filled up, Bgl II/Hind III sites of the pSuper.gfp/neo plasmid. Positive clones were verified by sequencing and used for transfections.

4.2.1.4 PCR-based mutagenesis for rescue constructs

In order to be able to control specificity of any phenotype arising in the knock-down cells, 'rescue' clones were established, in which expression of one of the dominant variants was restored. This was achieved by transfection of cDNAs for either CD44v4-v7 or CD44v6/v7, protected from degradation by four silent point mutations (highlighted in red) in the v7 target sequence, which were introduced by PCR.

v7 target site: AGAATGACAACACAGAGTCAAGAGGATG

v7 rescue site: AGAATGAC**T**AC**T**CAG**TC**TCAAGAGGATG

The amplificates were cloned into pCDNA3.1Hygro (Invitrogen, Karlsruhe) and positive clones were varified by sequencing. One of the stable knock-down clones, ASMLv71-14 was used for transfections with the 'rescue' constructs.

4.1.2.5 RNA-isolation and reverse transcription-PCR (RT-PCR)

RNA preparations were carried out with 'TRI Reagent' (Sigma, Seelze). About 5×10^6 cells were lysed directly into 1ml of TRI Reagent by scraping. After addition of 0.2ml chloroform and centrifugation (12000*g*, 15min, 4°C) the aqueous phase was transferred and

RNA was precipitated with isopropanol. After centrifugation the pellet was washed with ethanol, air-dried and resuspended in DEPC treated H_2Od. Integrity of the isolated RNA was controlled by gelelecrophoresis.

cDNA was generated using the 'ImProm II' system (Promega, Mannheim) with oligo (dT) primers following the manufacturer's instructions. 2µl of the reverse transcription reaction was used as template for the PCR.

4.2.2 Cell biology

4.2.2.1 Cell culture

Eukaryotic cells were kept in RPMI 1640-medium, containing 10% heat inactivated fetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin and cultured at 37°C, 95% humidity, 5% CO_2. For passaging, cells were trypsinized with 0.25% trypsin (w/v)/5mM EDTA in PBS (137mM NaCl, 8,1mM Na_2HPO_4, 2,7mM KCl, 1,5mM KH_2PO_4, pH 7.4).

4.2.2.2 Cryo-conservation of eukaryotic cells

$1x10^7$ cells were trypsinized, washed once with fresh medium and resuspended in ice-cold FCS/10% DMSO. Cells were kept over night at -80°C and transferred to liquid nitrogen.

4.2.2.3 Transfection of eukaryotic cells

ASML cells were seeded the day before transfection to 70% confluency. Transfection was carried out with the 'ExGene 500' reagent (MBI Fermentas, St.Leon Rot) following the instructions of the manufacturer. HEK293T cells were transfected with 'PolyFect Transfection reagent' (QIAGEN, Hilden) at 50% confluency according to the manufacturer's instructions.

4.2.2.4 Recloning of transfected cells by limiting dilution

Transfected cells were selected for drug resistance and checked by FACS for expression of the transgene. Limiting dilutions of 1 or 5 cells per well were carried out in 24-well plates. Cells were grown in the presence of $1x10^6$ freshly prepared rat thymocytes as

growth support. Clones were checked by FACS and used for a second round of dilution to ensure clonality.

4.2.2.5 Collection of conditioned cell culture supernatant

90% confluent cells were grown in serum free medium for 24h, the supernatant was harvested and centrifuged to remove cells (300g, 10min). Cell free supernatant was filtered through a 0.2µm filter and either coated to 24-well plates for adhesion assays, or concentrated through a ‘Vivaspin 6 column’ (50.000 MWCO) (Sartorius, Goettingen) and used for SDS-PAGE or gel filtration.

Deposited matrix was prepared by cultivating confluent cells for 24 hours and removing cells by EDTA treatment (5mM in PBS, pH8.0), followed by intensive washing with PBS.

4.2.2.6 Coating of plastic surfaces

Proteins bind under alkaline conditions to plastic surfaces. 50mM Tris (pH 9.5) was used as a binding buffer for matrix components, which were coated at a concentration of 10µg/ml. Collagens were pre-incubated at 37°C for 4h prior to coating. Hyaluronic acid was coated at very high concentrations (1mg/ml) under neutral pH in PBS. Conditioned cell culture supernatant was coated at pH 7.4. All coatings were carried out over night at 4°C, wells were washed once with PBS, followed by blocking with BSA (3mg/ml in PBS) for 2h at RT and three washes with PBS. Coated plates were used for adhesion assays.

Where indicated, conditioned cell culture supernatant was treated with hyaluronidase (Sigma, Seelze) or collagenase (Sigma, Seelze) for 4 h at 37°C prior to or after coating. Heat inactivated enzymes were used as controls.

4.2.2.7 Adhesion assay

Cells were trypsinized and recovered for 1-2h in RPMI/10% FCS. Adhesion assays were carried out in 24-well plates. Cells were washed with PBS, counted and $1x10^6$ cells were resuspended in serum free medium or PBS with or without additives. Cells were seeded and incubated at 37°C for 15min. Adherent cells were stained with crystal violet (see section 4.2.2.14).

4.2.2.8 Agglomeration assay

Cells were trypsinized and recovered for 1-2h and resuspended in conditioned cell culture supernatant of different origin. Where indicated, cell culture supernatant was treated with hyaluronidase (1mg/ml) at 37°C for 2 or 5h before use, or cells were seeded in RPMI medium, supplemented with 1mg/ml hyaluronic acid. Agglomeration was monitored with a Leica 'DM-IL' inverse microscope (Leica, Solms) and documented with a SPOT CCD camera using the SPOT 2.1.2 software.

4.2.2.9 Proliferation assay

$5x10^4$ cells were seeded in 96-well plates in RPMI supplemented with 10% or 0.5% FCS. ^{3}H-thymidin was added for 24h at different time points. Cells were harvested and H^3 incorporation was counted in a liquid scintillation counter. Alternatively $5x10^4$ cells were seeded as before and quantified by crystal violet staining after different time intervals (see section 4.2.2.14).

4.2.2.10 Soft agar assay

Tumour cells were suspended in RPMI/0.5% agar and either 100 or 1000 cells were seeded on a pre-poured RPMI/3% agar layer in the presence of 10% FCS. Colonies were counted after 4 weeks.

4.2.2.11 Drug treatment

$1x10^5$ cells per well were seeded in 96-well plates and grown overnight. Serial dilutions of a 50µg/ml starting concentration of cisplatin in RPMI medium were carried out in 1:2 steps. For chemical inhibitors starting concentrations were 100mM for the MEK1/2 and Akt II inhibitor and 200mM for the PI3K specific inhibitor LY294002 (Calbiochem, Darmstadt). Cells were treated for 3 days and surviving cells were stained either by MTT staining or crystal violet staining.

4.2.2.12 γ-irradiation of adherent cells

$1x10^6$ cells were seeded in 35mm petridishes, grown for 15, 24 or 48h, fresh medium was added and monolayers were subjected to different doses of γ-irradiation in a 'GAMMACELL 1000D' unit (AECL, Ontario, Canada). Survival was monitored by MTT staining after 72h. Where indicated, chemical inhibitors were added 30min prior to irradiation.

4.2.2.13 MTT staining of respiratory active cells

A 5mg/ml stock solution of MTT in PBS was diluted 1:10 directly in the growth medium of the cells and incubated for 30-40min at 37°C. Non-adherent cells were precipitated by centrifugation, the supernatant was aspirated and cells were resuspended in DMSO and measured at 550nm in an ELISA reader.

4.2.2.14 Crystal violet staining of adherent cells

Adherent cells were washed and fixed with 4% formalin in PBS for 4min at RT. The solution was changed to 1% crystal violet (in 10% EtOH) and incubated for 4min at RT. The plates were washed extensively with H_2Od, dried and cells were resuspended in 10% acetic acid. Absorbance was measured at 595nm in an ELISA reader.

4.2.2.15 FACS analysis

For FACS staining, cells were trypsinized and recovered in complete medium for 1-2h. Staining was performed in U-shaped 96-well plates with 10^6 cells per well. Incubation with primary and secondary antibodies in PBS was carried out at 4°C for 30min in the dark. After each step cells were washed three times with PBS by centrifugation at 300*g* for 4min. FACS analysis was performed using a 'FACSCalibur' (Becton Dickinson, Heidelberg) and analyzed with the 'CellQuest Pro' software.

4.2.2.16 Immunofluorescence staining of cells grown on coverslips

Cells were grown on coverslips for 1-2 days. Adherent cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 25min on ice, followed by

three washes with PBS/200mM glycine and three washes with PBG (PBS, 0.2% (v/v) gelatine, 0.5% (w/v) BSA). Primary antibody incubation was carried out for 1h at 4°C in PBG, followed by three washes with PBG. After incubation with Texas-Red conjugated secondary antibody for another hour at 4°C and three more washes with PBG, cells were rinsed with H_2Od, air-dried and mounted in Elvanol (20% (w/v) Mowiol in 2/3 PBS, pH 8.0 and 1/3 glycerine). Fluorescence microscopy was done using a Leica DMRBE microscope, equipped with a SPOT CCD camera using the SPOT 2.1.2 software for documentation.

4.2.2.17 Cryo-sectioning of tumour tissue

Tissues were embedded in frozen section medium 'Neg-50' (Richard-Allan Scientific, Kalamazoo, USA) and frozen in liquid nitrogen. Cryo-sectioning was carried out with a Reichert Jung '2800-FRIGOCUT E' to sections of 5μm thickness and transferred to chromalaune-gelatine coated glass slides for staining.

4.2.2.18 Immunohistological staining of cryo-sections

Sections were blocked with PBS/2% FCS for 30min, fixed with acetone/methanol (1:1) for 4min and washed with PBS. All incubation steps were carried out at 37°C in a humidity chamber. Sections were stained with primary antibodies or mouse IgG for 1h, after washing and incubation with biotinylated secondary antibody, detection was carried out using the 'Vectastain ABC kit' according to the manufacturer's instructions. Briefly, Vectastain AB-complex (containing avidin and biotinylated-peroxidase) was added for 30min, followed by incubation for 5-20min at RT with freshly prepared AEC mix [AEC-solution-1 (2.1ml acetic acid (0.1M), 7.9ml sodiumacetate (0.1M)) + AEC-solution-2 (4mg 3-amino-9-ethyl-carbazole (AEC) in dimethylformamide (DMF)) + 5μl H_2O_2 (30%)]. After washing with PBS, sections were counterstained with Mayer's Haemalaun and mounted in 'Kaisers glycerine-gelatine'. Microscopy was done using a Leica DMRBE microscope, equipped with a SPOT CCD camera using the SPOT 2.1.2 software for documentation.

4.2.3 Animal experiments

4.2.3.1 *In vivo* metastasis assay

$1x10^6$ tumour cells were suspended in PBS and injected into the footpad of 10-14 week old female BDX rats. Animals were sacrificed on day 50 in experiment 1 and on day 60 in experiment 2. Rats were dissected and diameters of primary and lymph node tumours were measured. Lungs were photographed and weighed. Samples of infiltrated tissues were embedded for cryo-sectioning and immunohistology or recultivated. For recultivation, tumour tissue was meshed through a sterile gauze and seeded in RPMI-medium.

4.2.4 Protein biochemistry

4.2.4.1 Surface biotinylation of molecules

Adherent cells were washed twice in PBS and incubated 30min at RT with 100-500µg/ml Biotin-X-NHS (Calbiochem, Darmstadt) in 25mM HEPES/150mM NaCl/5mM $MgCl_2$ on a shaking platform. Cells were washed three times with ice-cold PBS/200mM glycine and suspended in lysis buffer by scraping.

4.2.4.2 Immunoprecipitation (IP)

6x gel loading buffer: 300mM Tris pH6.8, 12% (w/v) SDS, 0.6% (w/v), bromophenolblue, 20% (v/v) glycerine

Lysis of cells was performed on ice or at 4°C and lysates were kept cold during the whole procedure. Cells were washed twice with PBS and scraped into ice-cold lysis buffer (25mM HEPES, 150mM NaCl, 5mM $MgCl_2$, 2mM PMSF, 1x proteinase inhibitor mix (Roche, Mannheim), 1% (v/v) detergent). Lysis was performed on a rotating platform for 1h. Unsolubilized material and cell nuclei were pelleted by centrifugation (15min, 15000*g*) and the cleared lysate was used for IP. Either 5µg purified antibody or 200µl hybridoma supernatant was used per 1ml cell lysate. Antibody binding was carried out for 1h on a rotating platform. For precipitations, 0.1 volumes protein G Sepharose was added to the antibody complexes and samples were rotated for another hour. Complexes were washed 4

times with lysis buffer. After the last washing step all liquid was removed through a 35g-needle attached to a vacuum line to ensure minimal background. Complexes were resuspended in gel loading buffer and boiled for 5min at 95°C. Sepharose beads were pelleted by short centrifugation and the supernatant was subjecetd to SDS-PAGE. For Re-IPs, the sepharose complexes were resuspended in lysis buffer containing a detergent of higher stringency (usually TX-100) and extracted for 1h at 37°C on a shaker. After removal of the sepharose, the supernatant containing the extracted antigens was subjected to another round of IP as described above.

4.2.4.3 Lysis of intact cells for SDS-PAGE

For western blotting of cell lysates from complete cells, cells were washed twice with PBS and scraped directly into gel loading buffer. Cell lysates were sonicated (5 impulses, 5sec each). Lysates were boiled for 4min and used for western blot analysis.

4.2.4.4 SDS-polyacrylamide gel electrophoreses (SDS-PAGE)

For electrophoretic separation of protein samples the 'Mini-Protean II' system from Biorad (Munich) was used for discontinuous SDS-PAGE. 5ml of separating gel (375mM Tris pH 8.8, 0.1% (w/v) SDS, 6-12% acrylamid-bisacrylamid, 0.1% (v/v) TEMED, 0.1% (w/v) ammoniumpersulphate) were overlaid with 2ml of stacking gel (375mM Tris pH 6.8, 0.1% (w/v) SDS, 5% acrylamid-bisacrylamid, 0.1% (v/v) TEMED, 0.1% (w/v) ammonium-persulphate). After complete polymerization, gels were loaded and run in gel running buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS) at a constant voltage of 200V. Gels were either stained with Colloidal Coomassie or silver or subjected to western blot analysis.

4.2.4.5 Western blotting (modified after Towbin et al., 1979)

After SDS-PAGE, protein gels were equilibrated for 10min in transfer buffer (25mM Tris, 192mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol). Nitrocellulose membranes (Amersham, Braunschweig) and 3MM whatman paper were equilibrated as well. For protein transfer, the gel was placed on whatman paper, followed by nitrocellulose and another whatman paper. The wet transfer was carried out in transfer buffer at a constant voltage of 30V over night at 4°C.

After transfer had been completed, the membranes were blocked for 1h at room temperature with 5% (w/v) fat free milk in PBST (PBS/0.1% (v/v) TWEEN 20) or, for detection with phosphospecific-antibodies, with 5% (w/v) BSA in TBST (TBS/0.1% (v/v) TWEEN 20). Antibody incubations were carried out for 1h at RT with hybridoma supernatant or purified antibody in PBST or TBST, respectively. Membranes were washed three times for 5min in PBST or TBST and incubated with secondary antibody conjugated to horseradish peroxidase (HRP) (diluted 1:5000 in PBST or TBST) for 1 h at RT, followed by additional three washing steps.

Biotinylated proteins were detected with ExtrAvidin-peroxidase (Sigma, Seelze). Detection was done by chemiluminescence using the 'ECL Western blotting detection reagents' and 'ECL radiography films' (both Amersham, Braunschweig).

4.2.4.6 Colloidal Coomassie staining of protein gels

After electrophoretic separation, proteins were fixed for 1h in 7% acetic acid/40% (v/v) methanol. Gels were stained over night in staining solution (4 volumes Colloidal Coomassie staining solution (Sigma, Seelze) + 1 volume methanol). Gels were destained for 30s in 10% acetic acid/25% (v/v) methanol and kept in 25% (v/v) methanol.

4.2.4.7 Silver staining of protein gels

After separation of proteins by SDS-PAGE, gels were fixed over night in 30% ethanol/10% acetic acid and sensitized for 45min (in 0.3% potassium tetrathionate, 0.5M potassium acetate, 30% ethanol), followed by 6 washes a 10min with H_2Od. Gels were stained with 0.2% silver nitrate for 1-2 h, rinsed with H_2Od and developed for up to 40min in developer (3% potassium carbonate, 31µl $Na_2S_2O_3$-$5H_2O$ (10%), 75µl formalin (37%) per 250ml). The reaction was stopped by adding 330mM TRIS/2% acetic acid and gels were kept in H_2Od.

4.2.4.8 Gelatine zymography for detection of MMP activity

Conditioned cell culture supernatant was collected as described and concentrated 10 times through a 'Vivaspin' column (50.000 MWCO) (Sartorius, Goettingen). The supernatant was mixed with Laemmli buffer, incubated for 15min at 37°C and subjected to SDS-PAGE

in an 8% acrylamide gel containing 1mg/ml gelatine as substrate. After electrophoresis the gel was incubated three times for 40min in 2.5% (v/v) TX-100, washed in developing solution (50mM Tris pH 7.4, 10mM $CaCl_2$, 150mM $NaCl_2$) and incubated for 24h in developing solution at 37°C and subsequently subjected to Coomassie staining.

4.2.4.9 Gel-filtration

Superdex 200/CL6B sepharose beads were washed in PBS/0.1% NaN_3, degased and packed into a column of 1.5cm diameter and 60cm length. The column material was equilibrated with PBS/0.1% NaN_3 over night. The void volume was calculated by blue dextrane. 30x concentrated cell culture supernatant was mixed with glycerine (900µl supernatant + 100µl glycerine) and loaded onto the column. Fractions of 3ml each were collected, of which 2ml were used for adhesion assays and 1ml was concentrated by TCA-precipitation and subjected to SDS-PAGE analysis.

4.2.4.10 Ultracentrifugation of cell culture supernatant

Cell culture supernatants were centrifuged at 100000*g* over night at 4°C in a Beckman Coulter 'Optima LE-80K' ultracentrifuge using a SW-41 rotor. The supernatant was transferred and precipitated material was washed with RPMI, centrifuged again for 1h and resuspended in RPMI medium. Supernatant and resuspended pellet was used for coating 24-well plates or subjected to SDS-PAGE.

4.2.4.11 TCA-precipitation of proteins

To concentrate protein in solution, proteins were precipitated with trichloric acid (TCA) at a final concentration of 5% (w/v) for 5min at 65°C and cooled on ice. After centrifugation (12000*g*, 30min, 4°C) the pellet was resuspended in gel loading buffer and subjected to SDS-PAGE.

4.2.4.12 Analysis of proteins by mass spectrometry

Protein gels were stained with Colloidal Coomassie as described. Proteins of interest were cut out with a scalpel. Subsequent preparations and mass spectrometrical analysis was

carried out at the central service of the DKFZ by MALDI-analysis (matrix assisted laser desorption/ionisation) using a 'Reflex II time-of-flight' mass spectrometer (Bruker-Daltonics GmbH, Bremen).

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Abbreviations

APC	allophycocyanin
APS	ammoniumpersulfate
AS	BSp73AS, rat pancreatic adenocarcinoma
ASML	BSp73ASML, rat pancreatic adenocarcinoma
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
CAM	cell adhesion molecule
CD44s	CD44- standard isoform
CD44v	CD44- variant isoform
cDNA	complementary DNA
Da	Dalton
DEPC	diethylpyrocarbonate
DISC	death inducing signaling complex
DKFZ	‚Deutsches Krebsforschungszentrum'
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DTT	dithiothreitole
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylendiamintetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EpCAM	epithelial cell adhesion molecule
ECM	extra cellular matrix
ERK	extracellular regulated kinase
pERK	phosphorylated ERK
FACS	fluorescence-activated cell sorter
FCS	fetal calve serum
Fig.	Figure
FITC	fluorescein isothiocyanate
g	gram
g	gravitational acceleration
G418	geneticine
GFP	green fluorescence protein
Gy	Gray
h	hour
H_20d	distilled water
HA	hyaluronic acid
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor (c-met)
HRP	horseradish peroxidase
hygro	hygromycine
ifp	intra-footpad
Ig	immune globulin
IP	immuno precipitation
i.v.	intra-venous

l	litre
k.d.	knock down
LB-medium	Luria-Bertani-medium
m	milli
µ	mikro
M	molar
MAPK	mitogen activated protein kinase
min	minute
MMP	matrixmetalloprotease
N-CAM	neural cell adhesion molecule
neo	neomycine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PMSF	phenylmethylsulfonylfluoride
POD	peroxidase
PVDF	polyvinylidenfluoride
Rb	retinoblastome
RNA	ribonucleic acid
RT	room temperature
s	second
ss	single stranded
ds	double stranded
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-poly-acrylamid-gelelectrophorese
siRNA	small interfering RNA
shRNA	short hairpin RNA
Tab.	Table
TCA	trichloric acid
TEMED	N,N,N`,N´,-tetramethylethylendiamine
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteases
TRIS	Tris(hydroxymethyl)aminoethane
TX-100	TritonX-100
TxRed	Texas Red
uPA	urokinase-type plasminogen activator
UTR	untranslated region
V	Volt
VEGF	vascular endothelial growth factor
v/v	volume/volume
WB	western blot
wt	wildtype
w/v	weight/vol