Metastasis-associated C4.4A acts as a linker between membrane proteases and alpha6beta4

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"*If you want to travel fast, walk alone; if you want to travel far, walk together*".

African saying

To my dear parents

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LIST OF ABBREVIATIONS

Acetyl-CoA
ADAM: A disintegrin and a metalloproteinase domain
APC: allophycocyanin
ARNT: aryl hydrocarbon receptor nuclear translocator
AS: BSp73AS, pancreatic carcinoma line
ASML: BSp73ASML, pancreatic carcinoma line
ATF: Cyclic AMP-dependent transcription factor
BSA: Bovine Serum Albumin
CAT: Chloramphenicol acetyl transferase
CBP: CREB binding protein
CD: Cluster of differentiation
CDKN2A: Cyclin-dependent kinase inhibitor 2A
CEBPβ: CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine) -enhancer-binding proteins
CIAP: Calf Intestinal alkaline phosphatase
CMV: Cytomegalovirus
$CoCl_2$: Cobalt chloride
CpG: C-phosphate-G
CREB: cAMP response element-binding
Cy2: cyanineDye2
d: day
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DPC4: Deleted in Pancreatic Cancer, locus 4
ECM: Extracellular matrix
EDTA**:** Ethylene diamine tetraacetic acid
EGF: Epidermal growth factor
EGFP: Enhanced green fluorescent protein
EGFR: Epidermal growth factor receptor
EMT: Epithelial-mesenchymal transition
FACS: Fluorescence-activated cell sorting
FCS: Foetal Calf Serum
FGF-2: Fibroblast growth factor-2
FIH: Factor inhibiting HIF
FITC: fluoresceinisothiocyanate

FN: fibronectin
GPI: Glycosyl phosphatidyl inositol
GTP: Guanosine triphosphate
h: hour
HCl: Hydrochloric acid
HE: hematoxilin-eosin
HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HB: heparin-binding
HIF: Hypoxia-inducible factor
HRE: Hypoxia response element
HRP: Horse radish peroxidase
IgG: Immunoglobulin G
IP: Immunoprecipitation
ifp: intrafoodpad
ip: intraperitoneal
KRAS: Kirsten rat sarcoma viral oncogene homolog
LN: laminin
LN1: laminin111
LN5: laminin332
LRP-1: Lipoprotein receptor-related protein 1
min: minute
mRNA: messenger Ribonucleic acid
MAPK: Mitogen-activated protein kinase
MMP: Matrix metalloproteinase
MT1-MMP: Membrane-type1-MMP; MMP14
OD: Optical density
PAGE: PolyAcrylamide Gel Electrophoresis
PBS: Phosphate buffered saline
PE: R-phycoerythrin
PHD: Prolyl-hydroxylase domain
PI: propidium iodine
PI3K: Phosphatidylinositol 3-kinase
PMA: phorbol 12-myristate 13-acetate
PMSF: Phenyl methyl sulphonyl fluoride
Prog: Progressor cells
Rpm: Revolutions per minute
RT: Room temperature

PTK7: Protein-tyrosine kinase-7
RANKL: Receptor activator of nuclear factor kappa-B ligand
RIPA: Radioimmunoprecipitation assay
ROS: Reactive oxygen species
RPMI: Roswell Park Memorial Institute
SD: Standard deviation
SDS: Sodium dodecyl sulphate
siRNA: small interfering RNA
STP: serine threonine proline
SOC medium: SuperOptimal with Catabolite repression
TACE: TNF-alpha converting enzyme, ADAM17
TAE: Tris acetate EDTA
TAPI: TACE inhibitor
Taq: *Thermus aquaticus*
TEMED: N,N,N´N´-Tetramethylene diamine
TEN: Tris EDTA NaCl
TGF: Tumor growth factor
TIMP: Tissue inhibitor of metalloprotease
TLC: Thin layer chromatography
TNF: Tumor necrosis factor
TP53: Tumor protein 53
U: unit
uPA: Urokinase-type plasminogen activator
uPAR: Urokinase receptor
VHL: von Hippel-Lindau
VEGF: Vascular endothelial growth factor
V/V: Volume/volume
WB: Western Blot
wk: week
W/V: Weight/volume
µM: microMolar
5-FU: 5-fluorouracil

1. INTRODUCTION

Cancer is the leading cause of death in the developed world and the second leading cause of death in the developing world, with approximately 13% of all deaths each year (Jemal, *et al*., 2011; WHO, 2006). Cancer cells, by definition, grow and proliferate in defiance of normal controls and are able to invade surrounding tissues and colonize distant organs. Cancer cells are thought to originate from a single cell that has experienced an initial mutation, but the progeny of this cell must undergo many further changes, requiring numerous additional mutations and epigenetic events, to become cancerous.

Tumor progression usually takes many years and reflects the operation of a Darwinian-like process of evolution, in which somatic cells undergo mutation and epigenetic changes accompanied by natural selection (Alberts *et al.*, 2008). The sequence of events underlying tumor progression and metastasis is subjected to perpetual elucidations and it is hoped that the study of cancer associated-molecules may provide data to better understand the cancer disease from the original disruption to the development of clinical symptoms.

Cancers are classified in two ways: by the type of tissue in which the cancer originates and by the site in the body, where the cancer first develops (www.cancercenter.com).

1.1 Pancreatic adenocarcinoma

Adenocarcinoma is a cancer that originates from an epithelium in glandular tissue. The pancreatic adenocarcinoma is the most common type of pancreatic cancer, accounting for 95% of pancreatic tumors. Less common types of pancreatic cancer include neuroendocrine or islet cell tumors. Pancreatic cancer is the fourth most common cause of cancer death across the world (Hariharan, 2008). Patients diagnosed with pancreatic cancer typically have a poor prognosis which is attributable to the fact that most patients have metastatic disease at the

time of diagnosis (Merl *et al*., 2010). Common symptoms include: pain in the upper abdomen, loss of appetite and/or nausea and vomiting, significant weight loss, painless jaundice, trousseau sign (in which blood clots form spontaneously in the portal blood vessels), diabetes mellitus, clinical depression, symptoms of pancreatic cancer metastasis (Pannala *et al.,* 2009; Carney *et al*., 2003).

The risk factors for pancreatic cancer include: cigarette smoking (approximately 30%), diet (a diet high in meat and fats increase pancreatic cancer risks), diabetes mellitus, chronic pancreatitis, family history, age, male sex, Helicobacter pylori infection (Raderer *et al*., 1998; Stolzenberg *et al.,* 2001; Michaud *et al*., 2007). Besides surgery, single agent gemcitabine is widely accepted as first-line therapy, common second-line chemotherapy regimens include oxaliplatin and 5-FU/leucovorin gemcitabine and oxaliplatin, oxaliplatin and capecitabine, and irinotecan-oxaliplatin (Brus and Saif, 2010).

The most frequently mutated oncogene in pancreatic cancer is KRAS2 which is mutated in >95% of pancreatic cancer (Hruban *et al.,* 1993). The KRAS2 gene is located on chromosome arm 12p and encodes a membrane-bound guanosine triphosphate (GTP)-binding protein. This GTP-binding protein mediates various cellular functions, such as proliferation, cellular survival, motility, and cytoskeletal remodeling (Hingorani *et al.*, 2003). Mutations in the KRAS2 gene are considered to be one of the earliest genetic events in pancreatic tumorigenesis (Jones. *et al*., 2008). Several additional signaling pathways downstream from KRAS2, including BRAF-MAPK and PI3K-AKT, may also be activated by mutations.

Three tumor suppressor genes, CDKN2A/p16, TP53, and SMAD4/DPC4, are commonly inactivated in pancreatic cancer (Caldas *et al.,*1994; Wilentz *et al*., 2000). These genes are inactivated by different mechanisms such as homozygous deletion of both alleles of the gene; intragenic mutation in 1 allele,

coupled with loss of the other allele; or promoter hypermethylation (Schutte *et al.*, 1997; Ueki *et al*., 2000).

Particularly in pancreatic cancer, the tumor microenvironment actively promotes invasion and tumor growth through a complex of interactions of different cellular components (Krautz *et al.*, 2011).

1.2. Tumor metastasis

Metastasis formation is a complex process which requires the dissociation of tumor cells from the primary tumor, migration through the extracellular matrix, penetration through the basal membrane, adaptation to the circulation pressure, docking to vascular endothelium, settlement and growth in distant organs (Fidler and Radinsky, 1990; Mareel *et al.,* 1991). These processes are responsible for ~90% of pancreatic cancer deaths in patients (DiMagno *et al*., 1999). Metastasis involves changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases (Keleg *et al.,* 2003).

Several controversial theories exist to explain the organ specificity of metastasis. Among them, the homing theory suggests that organs distant to sites of primary malignancy actively attract malignant cells via expression of adhesion receptors or by secretion of soluble chemotactic factors (Muller *et al.,* 2001). Identification of molecular addresses or adhesion receptors on endothelial cells in vascular beds of distal organs that specifically trap circulating malignant cells supports the active arrest view of the homing theory (Borsig *et al.,* 2002). In contrast, the "seed and soil" theory of Stephen Paget in 1889 stated that tumors do not metastasize randomly but rather at preferred sites. The formation of metastasis depends both on the properties of the tumor cells (the seed) as well as the permissive role of the environment (the soil) at the distant site (Fidler, 2003). Recently, it has been discovered that primary tumors themselves induce the formation of a suitable and appropriate environment in the organ where

metastasis will be seeded. Experiments have revealed that before the arrival of the metastatic tumor cells to a target organ, like the lungs or the liver, these organs are colonized by myelomonocytic cells originating from the bone marrow and mobilized by the primary tumor. These cells create an inflammatory-like microenvironment similar to the one formed in the primary tumor facilitating tumor cell seeding and initial survival in an *a priori* hostile environment. This has led to the concept of the premetastatic niche, whereby a special, permissive microenvironment in secondary target organs is induced over distance by the primary tumor. Some of the molecular mechanisms contributing to the formation of the premetastatic niche have been discovered and include mediators of inflammation, such as TNF, interleukins, matrix proteins or matrix-degrading enzymes (www.unifr.ch/pathology/en/ background/tumormetastasis). Tumor invasion encompasses the process of tumor cell penetration or infiltration into adjacent tissue. This event is also central to the development of metastasis. Loss of junctional contact between adjacent epithelial cells and cell-extracellular matrix association are essential prerequisites for tumor cell detachment from the primary tumor site (Tawil *et al*., 1996). It is postulated that migration and invasion of cancer cells into surrounding stroma are prevented by cell-cell and cell-matrix adhesion molecules. Disruption of these adhesive connections leads to increased motility of tumor cells, which detach from the primary lesion. Therefore, adhesion molecules on the cell surface play an important role in tumor cell migration and regulate the potential to metastasize (Keleg *et al.,* 2003).

Metastatic cancer cells may persist as small asymptomatic nodules for prolonged periods of time. This condition, also referred to as dormancy, has been reported in experimental models and patients. Dormant tumor cells were often observed in close proximity to quiescent vessels. This observation led to the hypothesis that dormancy is due to insufficient angiogenic capacity of the tumor cells, resulting in a state of balance between proliferation and cell death. Induction of

angiogenesis in dormant micrometastasis has been proposed as a mechanism triggering transition from a dormant metastasis to a growing metastasis. In addition, recruitment of bone marrow-derived or inflammatory cells has been reported to promote metastasis outgrowth. Thus, like in the primary tumor, changes in the microenvironment determine whether and when microscopic lesions will eventually grow to form a macroscopic metastasis. This proposed mechanism, although it helps to understand why metastases may appear many years after the primary tumor has been removed, does not yet explain why suddenly angiogenesis is induced and tumor growth is resumed. Nevertheless, dormancy is of great clinical relevance since it raises the possibility of therapeutic interventions before metastases resume growth (Bogenrieder *et al*., 2003).

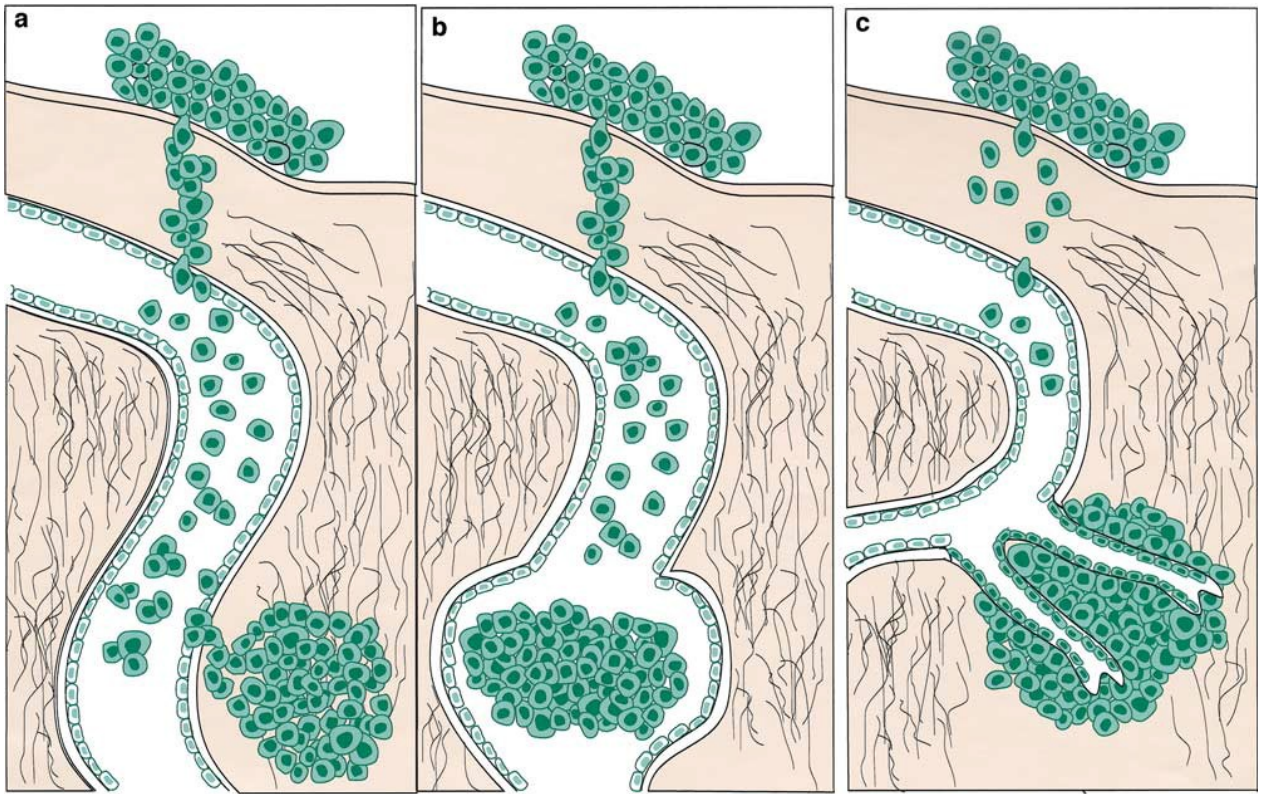


Figure 1: Models of metastasis. **(a)** According to Chambers and co-workers, only a very small population of injected cells (2%) form micrometastases, although over 87% are arrested in the liver. Furthermore, not all of the micrometastases persist, and the progressively growing metastases that kill the mice arise only from a small subset (0.02%) of the injected cells. **(b)** Muschel and co-workers recently proposed a new model for pulmonary metastasis in which endothelium-attached tumor cells that survived the initial apoptotic stimuli proliferate intravascularly. Thus, a principal tenet of this new

model is that the extravasation of tumor cells is not a prerequisite for metastatic colony formation and that the initial proliferation takes place within the blood vessels (Im *et al*., 2004). **(c)** The unique ability of aggressive tumor cells to generate patterned networks, similar to the patterned networks during embryonic vasculogenesis, and concomitantly to express vascular markers associated with endothelial cells, their precursors and other vascular cells has been termed 'vasculogenic mimicry' by Hendrix and co-workers (Hess *et al*., 2006).

1.2.1. Matrix metalloproteinases (MMPs) in metastasis

Matrix metalloproteinases (MMPs) are a family of highly homologous protein-degrading zinc dependent endopeptidases. This family currently includes more than 25 members that can be divided into collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and 9), stromelysins (MMP-3 and 10), matrilysins (MMP-7 and 26), and the membrane-type MMPs (MMP14 to 17 and -24, -25). Degradation and remodeling of the ECM, including the basement membrane, by proteolytic enzymes are essential steps in the process of cancer invasion, intra- and extravasation, and colonization at distant sites. Metalloproteases are thus important in many aspects of invasion and metastasis, ranging from cell proliferation and remodeling of the ECM to angiogenesis and cell migration. Most of these processes require a delicate balance between the functions of MMPs and ADAMs and tissue inhibitors of metalloproteases (TIMPs). ADAMs are named according to their structure, which includes a disintegrin and a metalloproteinase domain. ADAMs are membrane integrated proteases that regulate the function of a wide range of cell surface proteins by proteolytic ectodomain cleavage. Several of the ADAM proteins have alternative names, mostly given at their first description due to their preferential target, like ADAM17 that is also called TACE for TNF-alpha converting enzyme (Saftig and Reiss, 2011). TIMPs are a family of secreted proteins that selectively, but reversibly, inhibit metalloproteases in a 1 : 1 stoichiometric manner. During the invasive events, TIMPs are expressed primarily by the cancer cells and are thought to serve as a regulatory mechanism for fine tuning the activity of stromal MMPs, so that the cancer cells can have an active role in determining

where and when they invade (Chang and Werb, 2001; Egeblad and Werb, 2002). The expression and activity of MMP-2 and MMP-9, two intensively studied gelatinases, are frequently elevated in human cancer, which correlates with advanced tumor stage, increased metastasis, and poor prognosis (Lubbe *et al.*, 2006). MMP-3 is known to degrade collagen types III, IV, IX and X, proteoglycans, laminin, elastin, and fibronectin (Samnegard *et al.,* 2005). MMP14, also known as MT1-MMP, a key MMP that regulates invasion and metastasis, plays a dual role in pathophysiological digestion of the ECM through activation of proMMP-2 and direct cleavage of substrates such as collagen types I, II, and III. Regulated positioning of MMP14 to invadopodia, the specialized ECM-degrading membrane protrusions of invasive cells, enables focal degradation of ECM during invasion and metastasis (Poincloux *et al.,* 2009).

Degradation of structural and specialized components of the ECM by MMPs not only breaks the barrier that restrains tumor cell dissemination but also generates some bioactive fragments (Hua *et al.,* 2011). Upon MMP digestion, the ECM also releases biologically active fragments called matrikines, i.e., peptides originating from the fragmentation of matrix proteins and presenting biological activities (Ducaa *et al.,* 2004). For example, cleavage of laminin-5 γ2 chains by MMP-2 and MT1-MMP produces a fragment containing epidermal growth factor (EGF)-like motifs that engages EGFR signaling and larger fragments that engage integrin signaling, leading to cell migration (Koshikawa *et al*., 2005; Sadowski *et al.*, 2005). The cross-talk between MMPs and other proteases also contributes to tumor progression (Hua *et al.,* 2011). MMP-9 can regulate the activity of other proteases such as uPA. A recent study reveals that MMP-9 degrades the serpin protease nexin-1, an inhibitor of uPA. However, the effects of nexin-1 on tumor metastasis seem to be controversial. Nexin-1 reportedly binds low density lipoprotein receptor-related protein 1 (LRP-1) and stimulates extracellular signal-regulated kinase signaling, MMP-9 expression, and metastatic spread of mammary tumors (Fayard *et al.,* 2009). The inconsistency

of these studies is hard to explain. Given that MMP-9 can be upregulated by nexin-1, the degradation of nexin-1 by MMP-9 may represent a negative feedback regulation of nexin-1 activity (Xu *et al.*, 2010).

Except for components of the ECM, there are non-ECM substrates for MMPs that include growth factors, kinases, cytokines, chemokines, and receptors (Hua, *et al.*, 2011). MMP14 cleaves HB-EGF and removes the NH(2)-terminal 20 amino acids that are important for binding heparin. The truncated form of HB-EGF is independent of heparin and exhibits enhanced mitogenic activity (Koshikawa *et al*., 2010). Moreover, MMP14 degrades the Wnt/planar cell polarity protein-tyrosine kinase-7 (PTK7), an inhibitor of cell invasion. The cleavage of PTK7 by MMP14 leads to an increase in cell invasion and migration (Golubkov *et al.*, 2010). Tumor-associated MMP14 sheds RANKL and activates src-dependent prostate cancer migration and bone metastasis (Sabbota *et al.,* 2010). Thus, MMP14 may play pivotal roles in both the growth and metastasis of tumor cells (Hua *et al.*, 2011).

MMPs also have complex roles in angiogenesis. It is known that MMPs can promote endothelial cell migration and trigger the angiogenic switch. For example, MMP-9 participates in switching angiogenesis by releasing VEGF from ECM (Bergers *et al.,* 2000). Furthermore, MMPs increase the bioavailability of the pro-angiogenic growth factors vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and TGF-β, which stimulate proliferation and migration of endothelial cells. MMP14 regulates VEGF-α expression by promoting VEGFR-2 cell surface localization thereby activating the VEGFR-2-Src-Akt-mTOR pathway (Ito *et al*., 2009). However, MMPs may have adverse effects on angiogenesis. For example, MMP14-mediated endoglin shedding inhibits tumor angiogenesis (Hawinkels *et al.*, 2010).

Epithelial-mesenchymal transition (EMT) is a key developmental process characterized by loss of cell adhesion, repression of E-cadherin expression, and

increased cell mobility (Koshikawa *et al.,* 2010). EMT may be essential for numerous developmental processes including mesoderm formation and neural tube formation. During tumor progression, EMT is often activated to promote cancer cell invasion and metastasis. MMPs are important regulators or mediators of EMT. MMP-2 is necessary for the EMT that generates neural crest cells and plays an essential role inducing epithelial-mesenchymal transformations in the avian embryo (Duong and Erickson, 2004).

The overexpression and elevated activity of MMPs correlate with tumor progression. The balance between activated MMPs and their inhibitors such as TIMPs may define the net activity of MMPs. Down-regulation of TIMPs may result in an increase in the activity of MMPs and the invasive potential of tumor cells. Conversely, tumor invasion and metastasis can be inhibited by up-regulation of TIMPs in tumor cells (Hua *et al.,* 2011). Overexpression of TIMP-1 inhibits tumor growth and metastasis of melanoma (Khokha, 1994), suppresses human gastric cancer metastasis (Watanabe *et al*., 1996), and prevents oral squamous cell carcinoma progression (Wen *et al.,* 1999). Although accumulating data demonstrate that TIMPs have tumor-suppressive activities, studies also show that TIMPs may play contrasting roles in tumor progression (Hua *et al*., 2011).

1.2.2. Integrins

Integrins are non-covalently linked heterodimers of alpha and beta subunits (Gonzalez-Amaro and Sanchez-Madrid, 1999; Etzioni *et al*., 1999). They are transmembrane proteins that are constitutively expressed, but require activation in order to bind their ligand. To date, 15α subunits and 8β subunits have been identified. These can combine in various ways to form different types of integrin receptors. Integrins exhibit both “outside-in” and “inside-out” signaling properties (Coppolino and Dedhar, 2000; Zell *et al.,* 1999).

In addition to their roles in adhesion to ECM ligands or counterreceptors on adjacent cells, integrins serve as transmembrane mechanical links from those extracellular contacts to the cytoskeleton inside cells. The β4 subunit differs from all the others; its cytoplasmic domain being much larger, approximatively 1000 amino acids long instead of around 50, and making connections to intermediate filaments instead of to actin (Hynes, 2002). Alterations in α6β4-integrin-mediated signalling are accompanied by a reduction in cell motility, cell invasion, alterations in the organization of the actin cytoskeleton and changes in cell shape (Alam *et al*., 2011).

1.2.3 Hypoxia

The inner regions of malignant tumors become hypoxic as the tumor grows and rapidly expands. Hypoxia is a state where oxygen tension drops below normal limits and is a common feature of malignant tumors. Hypoxia is usually defined as ≤2% O_2, and severe hypoxia (or anoxia) is defined as ≤0.02% O_2. However, ambient air is 21% O_2 (150 mm Hg) and most mammalian tissues exist at 2%–9% O_2 (on average 40 mm Hg) (Bertout *et al.,* 2008). A key regulatory protein involved in the cellular adaptive response to hypoxia is hypoxia-inducible factor-1 (HIF1) (Vaupel *et al.*, 2004). The hypoxia-inducible factor (HIF) is a heterodimeric transcription factor consisting of one of three different oxygen-sensitive HIFα subunits (HIF1α, HIF2α, and HIF3α) and a common constitutive HIFβ subunit. Whereas HIF1αβ and HIF2αβ heterodimers function as transcriptional activators of oxygen-regulated target genes, the role of HIF3α is less clear, and a short splice variant of HIF3α, termed inhibitory PAS protein (IPAS), functions as a transcriptional repressor (Semenza *et al.*, 1999; Wenger, 2002; Seta *et al.,* 2002). HIF1β is constitutively expressed, but HIF1α has a short half life under normoxic conditions and is rapidly degraded. However, under hypoxic conditions, HIF1α is stabilized and induces the transcription of a

number of downstream target genes involved in physiologic and pathologic processes (Semenza, 2002; Maxwell *et al.,* 1997).

1.2.3.1. Regulation of HIF1α

The partial pressure of cellular oxygen is sensed by a family of prolyl hydroxylases that covalently modify HIFα subunits (Jaakkola *et al.,* 2001). Under normoxic conditions, HIFα is hydroxylated; hydroxylation promotes von Hippel-Lindau (VHL) tumor suppressor protein binding to HIFα, thereby targeting it for proteasomal destruction (Maxwell et *al.,* 1999). Under hypoxic conditions, the Prolyl-hydroxylase domain (PHD) activity (and thus HIFα hydroxylation) decreases. Thus, the high turnover rate of HIFα subunits enables the very rapid accumulation of HIFα under hypoxic conditions (Jewell *et al.*, 2001). Following a further decrease in oxygen availability, the asparagine hydroxylase function of the factor inhibiting HIF (FIH) also becomes impaired, resulting in a decrease in HIFα C-terminal hydroxylation. This decrease in C-terminal HIFα hydroxylation enables the increased recruitment of the p300 and CREB binding protein (p300/CBP) transcriptional coactivators, leading to the enhanced transcriptional activation of HIF target genes (Mahon *et al.*, 2001; Lando *et al*., 2002). Once the HIFα protein is stabilized, it heterodimerizes with the constitutively expressed HIFβ subunit, ARNT (aryl hydrocarbon receptor nuclear translocator), to form the heterodimeric transcription factor HIF. HIF binds DNA at hypoxia response elements (HRE) and recruits transcriptional coactivators such as p300/CBP. Apart from the essential protein-protein interaction between HIFα and HIFβ subunits, a multitude of other proteins are known to interact with HIFα (Wenger *et al.,* 2005).

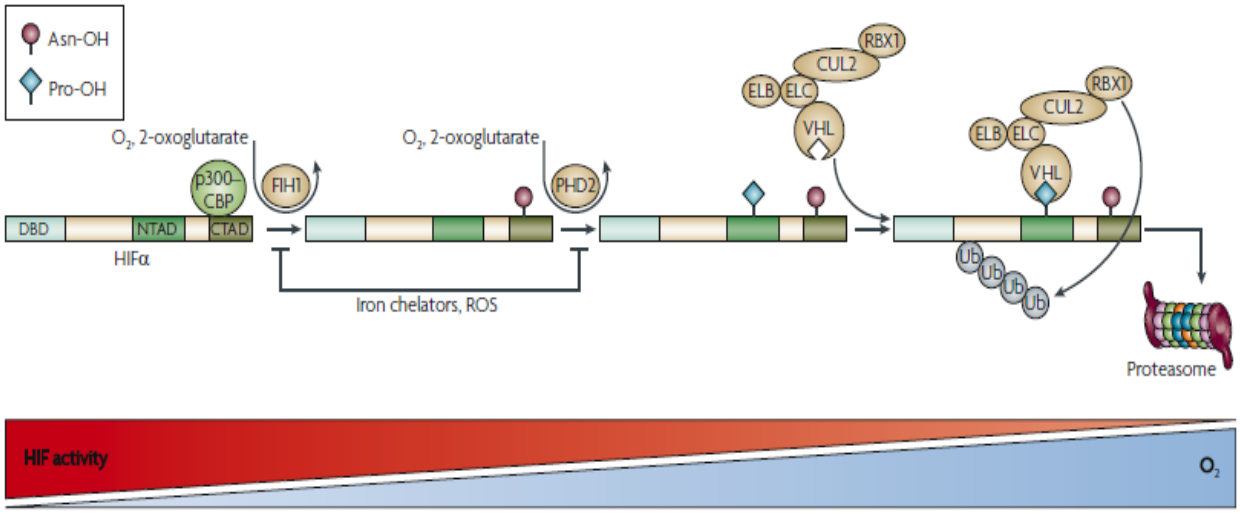


Figure 2: **Control of hypoxia-inducible factor (HIF) by hydroxylation.** HIF1α and HIF2α (shown generically as HIFα) contain a basic helix–loop–helix PAS DNA binding domain (DBD) and two transactivation domains (NTAD and CTAD). When O_2 levels are low, HIFα is stable and the NTAD and the CTAD are active. At intermediate levels of hypoxia the CTAD, especially of HIF1α, is hydroxylated on a conserved asparagine (Asn) residue by factor inhibiting HIF (FIH1). This prevents recruitment of the co-activators p300 and CREB-binding protein (CBP). As O_2 is more plentiful HIFα is hydroxylated on one (or both) of two proline (Pro) residues near the NTAD (for simplicity, only one site is shown) by prolyl hydroxlase 2 (PHD2), generating a binding site for the von Hippel-Lindau protein (VHL). Recruitment of the VHL ubiquitin (Ub) ligase complex containing elongin C (ELC), elongin B (ELB), cullin 2 (CUL2) and RING-box protein 1 (RBX1) leads to the polyubiquitylation and proteasomal degradation of HIFα. FIH1 and PHD2 require several cofactors, including 2-oxoglutarate and reduced iron, and are inhibited by reactive oxygen species (ROS) (Bertout *et al.,* 2008).

1.2.3.2. The consensus HIF responsive element (HRE) sequence

Once stabilized and activated, HIF binds to the consensus HRE, which is present in the oxygen-regulated elements of 70 known HIF target genes. Microarray experiments indicate that far more than 200 HIF target genes might exist; however, not all of these genes are likely to be directly regulated by an HRE in their regulatory regions. Rather, other HIF-dependent or -independent oxygen-regulated transcription factors might be responsible for their hypoxic induction. A single core HRE is necessary but not sufficient for efficient gene activation in response to hypoxia. Although the core HRE is the minimal DNA domain required for interaction with HIF, a fully functional HRE usually contains neighboring DNA binding sites for additional transcription factors. These

transcription factors are not necessarily hypoxia inducible, but they might amplify the hypoxic response or confer tissue-restricted activity to an HRE (Wenger *et al.,* 2005). For instance, HIF1 cooperates with ATF-1 and CREB-1 to transcriptionally activate the lactate dehydrogenase A gene (Ebert *et al.,* 1998; Firth *et al*., 1995) or with AP-1 binding factors to activate the gene encoding vascular endothelial growth factor (VEGF) (Damert *et al.,* 1997).

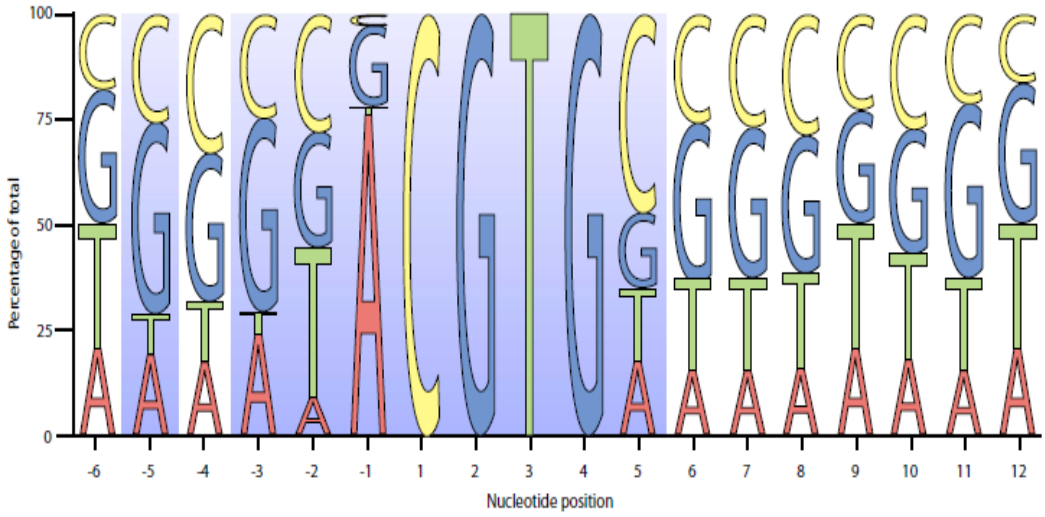


Fig. 3: The consensus core HRE sequence RCGTG (where R is A or G) and flanking nucleotides. Relative occurrence of the nucleotide distributions within 108 core HREs indicated as percentage of total. Shadowed boxes (light blue) indicate nonrandomly distributed nucleotide compositions, which occur mainly at 5′ flanking bases where HIFα subunits contact the DNA. For position −1, only one T and two C's were identified among 108 core HREs, raising concerns about their physiological relevance (Wenger *et al.,* 2005).

1.2.3.3. Mechanisms regulating HIF binding to the core HRE

Apart from the requirement for cooperation among transcription factors, epigenetic effects may also reduce the large number of putative core HREs to relatively few functional HREs. Methylation of the CpG dinucleotide (containing 5-methylcytosine) plays an important regulatory role in mammalian gene expression, contributing to X-chromosome inactivation and genomic imprinting, as well as tissue- and developmental stage-specific transcriptional regulation. CpG dinucleotides are underrepresented in the mammalian genome and are usually methylated if located outside of GC-rich "CpG islands." The

consensus core HRE contains a CpG dinucleotide, thus lowering the number of actual HREs found in the genome compared to the frequency of a random tetranucleotide sequence. Methylated CpG interferes with transcription factor binding to DNA through both direct steric hindrance and the binding of repressor proteins (Wenger *et al.,* 2005). It has been reported that HIF binding to the core HRE is blocked by 5-methylcytosine (Wenger *et al*., 1998).

Oxidative DNA damage caused by ROS might represent another epigenetic modification regulating HRE accessibility. Intriguingly, ROS induced in response to hypoxia oxidize particular bases within specific DNA sequences of the HIF target gene VEGF. The most frequently modified nucleotide is the terminal guanine of the VEGF core HRE (ACGTGGG). Because the base modifications occur close to, but not directly at the consensus core HRE, different HREs are likely to be unequally affected by ROS, providing a means of variable modulation of the efficiency of gene-specific transcriptional induction (Wenger *et al*., 1998).

1.2.4. Exosomes

Exosomes are small membrane vesicles, 30 to 100nm, of endocytic origin that are secreted by most cells in culture (Théry *et al.,* 2002). They derive from multivesicular bodies, which either fuse with lysosomes or fuse with the plasma membrane and release their intraluminal vesicles as exosomes. Thus, exosomes share the biochemical characteristics with the internal vesicles of the multivesicular bodies (Lakkaraju *et al*., 2008, Camussi *et al.,* 2010). The molecular composition of exosomes reflects their origin from intraluminal vesicles. Accordingly, the range of proteins recovered in exosomes is rather limited. Exosome preparations do not contain any proteins of nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus origin. All of the exosomal proteins that have been identified are found in the cytosol, in the membrane of endocytic compartments or at the plasma membrane. Besides

several common components, exosomes also contain cell type specific proteins. Exosomal proteins are functionally active (Théry *et al.,* 2002, Schorey and Bhatnagar, 2008, Zöller*,* 2009). Exosomes also contain mRNA and microRNA, which are transferred to the target cell, where they can be translated (mRNA) and mediate RNA silencing (microRNA) (Lakkaraju *et al*., 2008, Valadi *et al*., 2007). This process is target cell-specific, such that RNA is transcribed in one but not another type of cell (Valadi *et al*., 2007). Thus, exosomes constitute a potent mode of intercellular communication that is important in immune response (André *et al*., 2002; Chaput and Théry, 2011), cell-to-cell spread of infectious agents (Schorey and Bhatnagar, 2008) and tumor progression (Zöller, 2006).

The mode whereby exosomes interact with their target has not yet been fully elucidated. Exosomes can bind to target cells, fuse with the target cell membrane or be taken up by the target cell. These modes of interaction are not mutually exclusive. It is, however, important to note that the exosome target cell interaction is selective. This is very important, as exosomes by the transfer of proteins, mRNA and microRNA can severely affect the fate of the target cell (Théry, 2011; Mittelbrunn *et al*., 2011; Valadi *et al*., 2007).

1.3. The BSp73 tumor model

The BSp73 tumor was diagnosed as a pancreatic adenocarcinoma in the BDX rat strain with a local tumor in the pancreatic tissue and ascitic metastasis. When implanting the local tumor subcutaneously, it maintained its local growth behavior and grew as a solid tumor at the implantation site (AS). Instead, when the tumor cells derived from the ascites were subcutaneously implanted, no local growth was observed. Instead, the tumor cells metastasized via the lymphatic system to the lung, where they formed thousands of miliary metastases (ASML) (Matzku *et al.,* 1985). The highly metastasizing variant ASML showed spherical morphology in culture, while the nonmetastatic variant AS showed adhesion and

spreading via vinculin-containing focal contacts. These cells also synthesize, secrete and assemble fibronectin at the pericellular area. The metastasizing ASML variant cells adhered to the substrate at a slower rate via thick cytoplasmic protrusions, but are removed from the substrate by trypsin-EDTA slower than the non-metastasizing AS variant cells. The ASML cells also synthesize very low levels of both vinculin and fibronectin, display a diffuse pattern of actin and tubulin organization, and are unable to spread on substrates. Spreading could not be induced in the ASML cells by seeding the cells on an extracellular matrix derived from bovine corneal endothelial cells or on concanavalin A (conA)-coated substrates, or by the addition of db-cAMP to the medium (Matzku *et al*., 1983; Raz *et al*., 1985). To further define differences between the two sublines, monoclonal antibodies were generated and selected for those that only bind to the metatastasizing ASML cells (Matzku *et al*., 1989). With the help of these monoclonal antibodies, the genes of suggestedly metastasis associated molecules were identified. One of these molecules is C4.4A.

1.4. C4.4A: A metastasis-associated molecule

C4.4A is a 65-100 kDa glycosylphosphatidylinositol (GPI)-anchored molecule, which shows 46.9% homology to uPAR (Rösel *et al.*, 1998). It belongs to the Ly6/uPAR/alpha-neurotoxin (LU) protein domain family. The gene coding for human C4.4A is located on chromosome 19q13. It is composed of 4870 bp and 5 exons. A putative role of C4.4A in cancer invasion and metastasis was emphasized by the finding that only metastasizing rat carcinoma expressed C4.4A (Claas *et al*., 1996; Rösel *et al*, 1998). Cloning of human C4.4A confirmed preferential expression in metastatic tissue (Würfel *et al*., 2001).

1.4.1. The C4.4A protein

C4.4A consists of 346 amino acid residues, including a 30 residues signal peptide at the N-terminal and a C-terminal signal sequence for GPI anchorage (38 residues) that are cleaved post-translationally, yielding a mature protein of 278 residues, anchored to the cell membrane via GPI. It contains two LU domains (domains I and II), each of about 90 amino acids, and a serine-, threonine-, proline-rich (STP-rich) region. LU domains adopt a "three-fingered" folding topology that is characterized by 4 consensus disulfide bonds and an invariant C-terminal asparagine. Intriguingly, domain I of C4.4A lacks one consensus cysteine bond, which is crucial to the proper folding of the single domain LU proteins (Jacobsen and Ploug, 2008). The STP-rich region is highly O-glycosylated, with 17 potential O-glycosylation sites. None of the 6 potential N-glycosylation sites of C4.4A are, however, located in this region (Rösel *et al,* 1998; Jacobsen and Ploug, 2008). Different degrees of glycosylation can probably explain the large variation in molecular weight observed in C4.4A from different sources (Paret *et al*., 2007; Hansen *et al*., 2004), deviating from the theoretical value of 36 kDa (Jacobsen and Ploug, 2008).

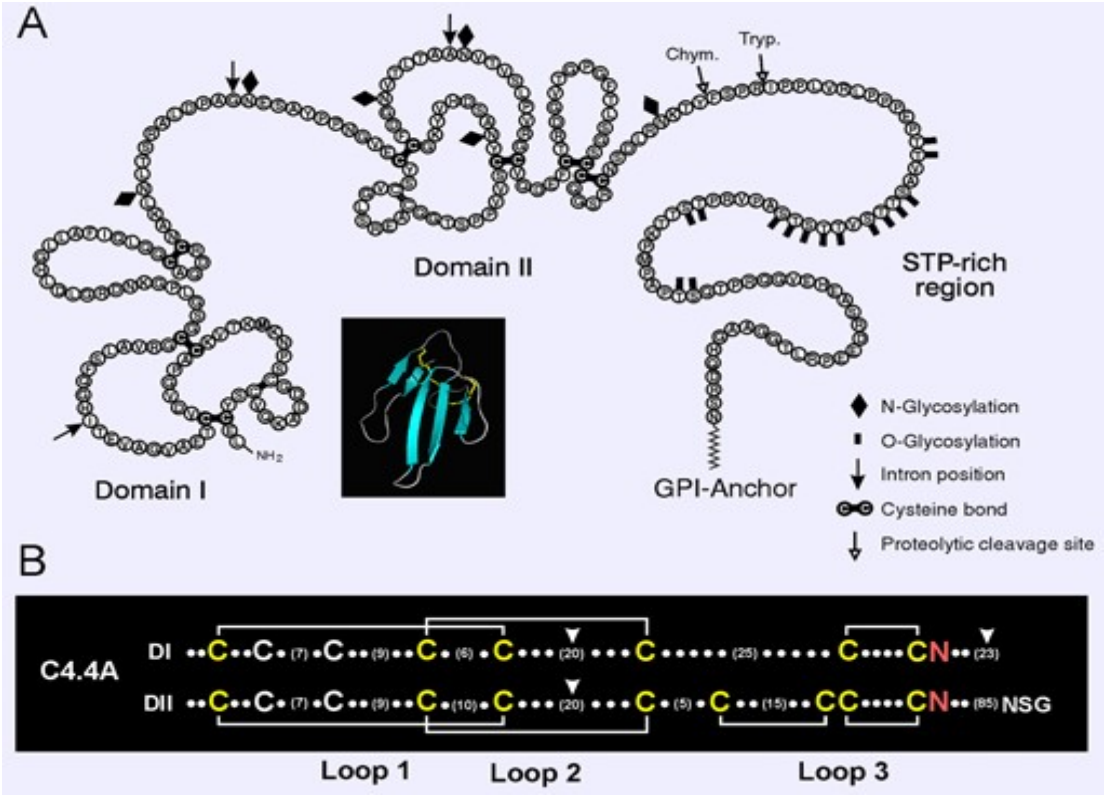


Figure 4: Protein structure of C4.4A. (**A**) Structural representation of the two LU domains and the STP-rich region of C4.4A (modified from Hansen et al., 2004). Insert: Ribbon diagram of the three-finger fold of a single LU domain (made in PyMOL™(DeLano Scientific), using PDB coordinates 1NEA). (**B**) Disulfide connectivity in C4.4A, with LU consensus cysteine bonds highlighted in yellow (Jacobsen and Ploug, 2008).

1.4.2. Expression of C4.4A

Transcription of the *C4.4A* gene requires the contribution of the transcription factor C/EBPbeta. This transcription is strongly enhanced by JunD and c-Jun such that *C4.4A* is even transcribed in a C4.4A-negative tumor cell line after cotransfection with C/EBPβ plus JunD or c-Jun (Fries *et al*., 2007).

Human and rat C4.4A have been demonstrated to be expressed in several types of carcinoma like mammary, renal cell, colorectal (Smith *et al.,* 2001; Würfel *et al.,* 2001; Seiter *et al*., 2001; Fletcher *et al*., 2003; Hansen *et al*. 2004; Paret *et al*., 2007) and most pronounced non-small cell lung cancer (Hansen *et al*., 2007). Its expression in other types of cancer, like esophageal cancer and malignant melanoma becomes regulated during tumor progression (Seiter *et al.*, 2001; Hansen *et al*., 2008; Wang *et al*., 2006). C4.4A is not expressed on leukemia and lymphoma (Würfel *et al.,* 2001). However, weak C4.4A expression already at the stage of hyperplasia suggests that C4.4A may also be an early biomarker for a possibly more malignant subtype of adenocarcinoma (Jacobsen *et al.,* 2011).

Besides on malignant tumors C4.4A expression has been observed in the rat only on basal to suprabasal layers of keratinocytes, squamous epithelia of the upper gastrointestinal tract, urothelium and in placental tissue. In the adult mouse, a very similar expression profile has been seen: suprabasal layers of the squamous epithelia of the oral cavity, esophagus, non-glandular portion of the rodent stomach, anus, vagina, cornea, and skin (Claas *et al*. 1996; Rösel et al., 1998; Smith et al, 2001; Hansen *et al*., 2004; Kriegbaum *et al*., 2011). Though C4.4A is tethered to the cell membrane via a GPI-anchor, it can under certain conditions also be found intracellular. Tumor cell lines release C4.4A by vesicle shedding and proteolytic cleavage (Paret *et al.,* 2007). A soluble fragment of C4.4A, termed C4.4A', resulting from cleavage in the protease-sensitive region between domain II and the STP-rich region, releasing the two N-terminal LU domains, has been described in esophageal tissue (Hansen *et al*., 2008).

1.4.3. Function of C4.4A

Despite the structural homology of C4.4A to the urokinase receptor (uPAR), there is no evidence for shared functions. uPAR has a well-established role in regulating and focalizing uPA-mediated plasminogen activation to the surface of those cells expressing the receptor. Instead, the biological function of C4.4A remains elusive. Circumstantial evidence, nevertheless, points to a role of C4.4A in the modulation of cell-cell and/or cell-matrix interactions (Rösel *et al,* 1998; Paret *et al*., 2005; Hansen *et al*., 2008). Association of C4.4A with LN1 (also known as LN111) and LN5 (alternative name LN332), induces spreading, lamellipodia formation and migration. The carbohydrate-binding protein galectin-3, which has been reported to be involved in cell/cell interactions, cell adhesion, migration, invasion and metastasis, has also been identified as a ligand for C4.4A (Paret *et al*., 2005). C4.4A and the cell adhesion molecule E-cadherin are co-expressed in the normal esophageal mucosa, and both are down-regulated in the progression to dysplasia (Hansen et al., 2008). C4.4A-positive and not C4.4A-negative tumor cells are capable of penetrating a matrigel, and this process can be inhibited by a monoclonal anti-C4.4A antibody. Encapsulation of lung metastases in rats, arising after an intrafootpad injection with pancreatic tumor cells, disappears, when these tumor cells are transfected with C4.4A (Rösel *et al*., 1998). C4.4A has been reported to be a novel substrate for the extracellular matrix-degrading metalloproteases ADAM10 and ADAM17, which have been implicated in cell migration and proliferation, with a bearing on tumor invasion and metastasis (Esselens *et al*., 2008).

Taken together, there is circumstantial evidence for C4.4A being engaged mostly in cell matrix interaction and possibly matrix degradation, without insight into the molecular mechanism.

1.5 Aim of thesis

C4.4A was first identified in a screening designed to select membrane proteins overexpressed in metastasis. Due to its very restricted expression in non-transformed tissue and its well defined overexpression in several epithelial tumors, C4.4A offers itself as a diagnostic marker. Its potential use as a therapeutic target is hampered by the lack of knowledge about its functional activity. I approached this open question, by focusing on the previously reported connection between C4.4A upregulation in wounded tissue as well as in tumors in hypoxia. Central to my work were three questions:

1. Does hypoxia regulate C4.4A transcription?

It has been reported that C4.4A transcription requires CEBPβ and is supported by JunD and c-Jun. I focused on HIF1α, one of the main transcription factors regulating hypoxia-induced genes. HIF1α could indeed be engaged in C4.4A transcription, as the C4.4A promoter contains 3 HIF responsive elements.

2. Does hypoxia affect the interaction of C4.4A with the extracellular matrix?

It has been established that C4.4A binds to laminins 1 and 5. It also is known that C4.4A transiently contributes to laminin adhesion, whereas it promotes motility on laminin for a long time. However, the underlying molecular mechanims are unknown. Based on the results of these studies, I wanted to confirm

3. the *in vivo* relevance of C4.4A in the metastatic process.

To approach this last question, I generated a C4.4A knockdown of the metastatic rat ASML tumor line, whose growth behavior and functional activities were compared *in vivo* and *in vitro* with that of the parental line.

C4.4A responds to hypoxia, though not at the transcriptional level. It mostly acts as a molecular facilitator by recruiting the α6β4 integrin and by MMP14 activation. Thereby cell bound, but also exosomal C4.4A promotes tumor cell

motility and invasiveness. Via the association with α6β4 it also becomes engaged in apoptosis resistance.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Name	Company
Agitator for bacterial cultures	Edmund Buehler GmbH, Hechingen
ELISA Reader	Thermo Scientific, Langenselbold
Camera system Spot CCD	Diagnostic Instruments, Sterling Heights, USA
Cell chamber Neubauer improved	Brand, Wertheim
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Hanau
DNA-agarose gel electrophoresis chamber	Bio-Rad, Munich
FACS Calibur	Becton-Dickinson, Heidelberg
Fuji Film Imaging Plate BAS-MS 2325	Fuji Photo Film, Düsseldorf
Hyper processor (for processing films)	Amersham, Freiburg
Incubator for bacteria	Melag, Berlin
Incubator for cell culture	Labotec, Goettingen
Master cycler (PCR cycler)	Eppendorf, Hamburg,
Magnetic stirrer 3000	Heidolph, Keilheim
Microscope DMBRE	Leica, Bensheim
Microwave	Phillips, Wiesbaden
Phosphorimager Fuji Film BAS-1800II	Fuji Photo Film, Düsseldorf
Photocassette	Amersham, Freiburg
Ph-Meter-761 Calimatic	Knick, Berlin
Photometer Ultraspec III	Amersham, Freiburg
Pipettus-Akku	Hirschmann, Eberstadt
Pipettes	Eppendorf, Hamburg
Power supply PS 9009	GIBCO, Darmstadt
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA

Name	Company
Rotor SW41 Ti	Beckman Coulter, Krefeld
Speedvac centrifuge	Bahofer, Reutlingen
Sterile hood	Heraeus, Hanau
Sonicator Sonoplus	Bandelin, Berlin
Table top centrifuge	Heraeus, Hanau
Transfer apparatus Mini Trans-Blot®	Bio-Rad, Munich
Thermo-mixer	Eppendorf, Hamburg
Ultrasound homogenizer	Bandelin Electronik
Water-bath	Julabo, Seelbach
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortex Genie	Si Inc., New York, USA

2.1.2. Miscellanoeus materials

Name	Company
Cell culture flasks 25cm^2, 75cm^2	Greiner, Frickenhausen
Cell culture 96-well, 24-well, 6-well plates	Greiner, Frickenhausen
Centrifugal concentrators Vivaspin 6ml, 20ml	Vivascience, Hannover
Cryovials	Greiner, Frickenhausen
Coverglass	R. Langenbrinck, Emmendingen
Dako pen	DakoCytomat., Glostrup, Denmark
Electroporation cuvettes	Eugentec, Seraing, Belgium
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen
Glass slides	R. Langenbrinck, Emmendingen
Hyperfilm ECL	Amersham, Freiburg
Needles	BD Biosciences, Heidelberg,
Nitrocellulose membrane Hybond ECL	Amersham, Freiburg
Parafilm	Greiner, Frickenhausen
Petridishes	Greiner, Frickenhausen
Pipette tips	Sarstedt, Numbrecht

Name	Company
Sterile filter 0.2µm	Renner, Darmstadt
Syringes	BD Biosciences, Heidelberg
Trans-well migration (Boyden) chambers 48 well	Neuroprobe, Gaithusberg, USA
WhatmanTM 3MM paper	Scleicher & Schüll, Dassel

2.1.3. Chemicals and reagents

Name	Company
Acetic acid	Riedel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Ampicillin sulphate	Calbiochem, Darmstadt
Bactoagar	Fluka, Buchs, Switzerland
Bio-Rad, Munich Bradford reagent	Bio-Rad, Munich
Bovine Serum Albumin (BSA)	PAA, Pasching, Austria
Bradford	Sigma, Steinheim
Brij 96	Fluka, Buchs, Switzerland
Bromo phenol blue	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
Chloroform	Riedel-de Haen, Seelze
D-*threo*-Chloramphenicol- (dichloroacetyl-1-^{14}C)	Sigma, Steinheim
Coomassie R-250	Merck, Darmstadt
Crystal violet	Sigma, Steinheim
Dimethyl formamide	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Ethanol	Riedel-de Haen, Seelze
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacitic acid (EDTA)	Sigma, Steinheim
Foetal Calf Serum (FCS)	PAA, Pasching, Austria
Formaldehyde (37%)	Merck, Darmstadt

Name	Company
G418 sulphate	PAA, Pasching, Austria
Gelatine (cold water fish skin)	Merck, Darmstadt
Glucose	Merck, Darmstadt
L-Glutamine	AppliChem, Darmstadt
Glycerine	Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden
Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hygromycin	PAA, Pasching, Austria
Immersion oil	Zeiss, Goettingen
Isoves DMEM Medium	Invitrogen, Darmstadt
Lipofectamine	Invitrogen, Darmstadt
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
Methanol	Riedel-de Haen, Seelze
N,N,N´N´-Tetramethylenediamine (TEMED)	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
PMA	Sigma, Munich
Potassium acetate	Sigma, Steinheim
Potassium carbonate	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Potassium tetrathionate	Merck, Darmstadt
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg

Name	Company
Puromycin	Calbiochem, Darmstadt
RPMI 1640	GIBCO, Darmstadt
Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
Sodium hydroxide	Riedel-de Haen, Seelze
Tris	Roth, Karlsruhe
Tritic YG	AppliChem, Darmstadt
Triton-X-100, Triton-X-114	Sigma, Steinheim
Trypan bue	Serva, Heidelberg
Trypsin	Sigma, Steinheim
Trypton	AppliChem, Darmstadt
Tween 20	Serva, Heidelberg
Yeast Extract	GIBCO, Darmstadt

2.1.4 Buffers and solutions

Bicarbonate buffer	15mM Na2CO3, 35mM NaHCO3, pH 9.6
Blot buffer	25 mM Tris, 192mM Glycine, 0.1% SDS, 20% Methanol
Ethidium Bromide	0.01% (w/v) in water. Store in dark.
Freezing medium	10% DMSO in FCS
HEPES buffer	25mM HEPES, 150 mM NaCl, 5mM MgCl2, 1 mM PMSF, Protease inhibitors
6x Laemmli-buffer	350mM Tris, pH6.8, 10% (w/v) SDS, 36% (w/v) Glycerine, 0.01% (w/v) Bromophenol blue

LB medium	10g peptone, 5g yeast extract, 10g NaCl. Make volume to 1l. Add 15g agar for LB plates
Running buffer (10X)	1%SDS (w/v), 144g Glycine, 30g Tris. Make volume to 1l with bidestilled water
PBS	137 mM NaCl, 8.1mM Na2HPO4, 2.7 mM KCl, 1.5mM KH2PO4, pH 7.4
RIPA buffer	25 ml Tris.HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS
Stripping buffer	62.5 mM Tris-HCl (pH 6.8), 2% SDS ,0.1 M, 2-Mercaptoethanol
TAE buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5M EDTA pH 8.0. Make volume to 1l and adjust pH to 8.5
TEN buffer	40mM Tris/Cl pH 7.5, 1 mM EDTA pH 8.0, 150 mMNaCl

2.1.5 Enzymes

Name	**Company**
Restriction enzymes	MBI Fermentas, St. Leon-Rot, Germany
Taq polymerase	MBI Fermentas, St. Leon-Rot, Germany
T4 Ligase	MBI Fermentas, St. Leon-Rot, Germany
Calf Intestinal alkaline phosphatase (CIAP)	MBI Fermentas, St. Leon-Rot, Germany

2.1.6 Kits

Name	**Company**
Qiaquick gel extraction kit	QIAGEN, Hilden, Germany
Qiaquick Midiprep kit	QIAGEN, Hilden, Germany
ECL Western Blotting Detecting Reagent	Amersham, Freiburg, Germany

2.1.7 Markers

Name	Company
GeneRulerTM 100bp and 1Kb DNA-Ladder Plus	MBI Fermentas, St. Leon-Rot
PagerulerTM Prestained Protein Ladder	MBI Fermentas, St. Leon-Rot

2.1.8 Antibodies

2.1.8.1 Primary antibodies

Antibody	Provider / Company
C4.4 (anti-rat C4.4A)	Matzku *et al*., 1989
D5.7 (anti-rat EpCAM)	Matzku *et al*., 1989
B5.5 (anti-rat α6β4)	Matzku *et al*., 1989
anti-human C4.4A	Paret *et al.,* 2007
anti-rat panCD44 (clone Ox50)	European Association of Animal cell cultures, Porton Down, UK
anti-rat uPAR	American Diagnostica, Pfungstadt
anti-rat uPA	American Diagnostica, Pfungstadt
anti-rat TACE	Stressgen, Ann Arbor, MI, USA
anti-rat MMP14	Santa Cruz Biotech, Heidelberg
anti-rat HIF1α	Santa Cruz Biotech, Heidelberg
anti-rat β4	BD, Heidelberg
anti-actin	BD, Heidelberg
anti-fibronectin	BD, Heidelberg
anti-rat LN1	BD, Heidelberg
anti-human LN5 (crossr. rat)	BD, Heidelberg
rLS3 (anti-human LNα3 chain)	Nakashima Y *et al*., 2005
8A5212 (anti-hu.LNβ3 chain)	Nakashima Y *et al*., 2005
D4B5 (anti-human LNγ2 chain)	Nakashima Y *et al*., 2005

2.1.8.2 Secondary antibodies

Antibody	Company
anti-mouse IgG-APC	BD, Heidelberg, Germany
anti-mouse IgG-HRP	Dianova, Hamburg, Germany
anti-mouse IgG-PE	Dianova, Hamburg, Germany
anti-rabbit IgG-HRP	Dianova, Hamburg, Germany
anti-hamster IgG	Dianova, Hamburg, Germany
Streptavidin-HRP	Sigma, Steinheim, Germany

2.1.9 Matrix proteins

Protein	Company
Fibronectin (2µg/ml)	Sigma, Munich, Germany
LN1(5µg/ml)	Sigma, Munich, Germany
rat LN1	Sigma, Munich, Germany
human LN5	A431 exosome-depleted culture supernatant w/o FCS
rat LN5 (50µg/ml)	804G exosome-depleted culture supernatant w/o FCS

2.1.10 Inhibitors

Inhibitor	Company
Aprotinin (serine protease inhibitor) (10µM)	Sigma, Munich, Germany
MMP9/13-Inhibitor-II (MMP-Inh.II) (3µM)	Merck, Darmstadt, Germany
TAPI (TACE Inhibitor) (40µM)	Merck, Darmstadt, Germany

2.1.11 Nucleic acids

2.1.11.1. HIF responsive elements (HRE) in the *C4.4A* promoter

TTATTTTTTTCTTCAAGCTCTGGGGATAGGATCAGGGCTTCATCCACACCAGGCAAACCATCAAAC
TTCCCTTTTTCATTTGGAAGACTGCCCAGTGACCCCAGTCTTCCTGCGCCCTTTATCAAGCCTGGGG
AGGAGTTTGAAATAGGGTCTCACTATGTAGCTCAGGCTGGCTTCAAACTCAATACAATCCTGTCTG
AGCTTCCTAAGTCTAGAATTTTAAGCAGGAGCCATTATACCCAGCCCTCCCTTCCTCCCTCTCCCTC
CCTGCCCTCCATTCTTGTTTTTATCACTCTTGATTTCTCTGCAGGTTCATTGTCTAGGGTCTGTGATT
CTGTCTCATTACATTTATTTATTTATTCATTACTTGGAGTTGTGGTACAGATATGTGGAAGGCAGAA
GATACTTCTTAGGAATCAATTCTTGTGTCTCTCTTTCTACCATGTGGGTTCCAGGGATCCAACTCGG
GCCCTCAGGTTGGAGACAGGCGTTTTTACATGCGAACCATGTCACACACACACACACACACACACA
CACACACACACACCCATATACAGTCTCTCTATGCAGCCCAGACTCCGGTCAAACTTAG
GTTCTGTCTTGGCCTCCTA**CGTG**CTGAACTACAGGGAAGCCCTGGCTCAGCTTCCCAATGTACAA
CCAGTGAAGATCTAAGTATGCGGCCGGCGATGCTGTAGATTGTACATCACAGGCACACACACAAG
GCCACCCTCCCTAGAAGGAAATGGAAGAGGCATCTTCCAGGACAAGACTCTCTGAGAGGGAACCT
CTATTCGTTTTCTAAACTGACCCATTTCTGTGGGTTAGATTGGACTGTTTCAGAACTCCACATAAAA
GTCACACTGTGCGCTGCCTTGCTTCTGGTTTCATCGAGTCATTGTTTTAAAGATCCATCTATGTAGCT
GTATACACCATCCACTGAAACCTTCCATTGCTAAGCGACAGTGCATGGGGTGGAGGGACC
ACT**CGTG**GTTTTATTACTCAATTAATTAATTATTTATTGAATCAGGCGATGTTGGGGATGGACCCC
AGGGCTTCGTTCATGCTGAGTGAGTGCTTTACCACTAAGCCTTAAGTACTACCATCTCTCACTGAGG
GAATCTAGGCAGGGGCTCTACCACTGAGCCACGCCCCCAGCCCCTCACTGGGGGATTCTAGGCAGG
GGCTCTACCACTGAGCCACGCCCCCAGCCCCTCACTGGGGGATTCTAGGCAGGACTCCACTGCTGA
CCCCTAATGCACTATCCGAACTCTCCAACCACTATTTTTTTTTTTAATTTAAAGGACTTATTAGAGTG
CCCAGCACACATGATAGTTGTGCAAGACTTGTTTTCATTTTTTATTCATTTTTTGAGACTTTCAGCCA
TGTTTGCAATGTATTTTGATCTATATACCTTTCTTATCACCTCTCGATAGATACCCGATATT
ATTTCCCAGCTTAATCATATTGAAAAGTCTACTTCTCAATTTCAGCTCGA**CGTG**TAGCCCTTTATC
CGTATTCCTCCACAGCCTTAATCTCTTGTAAGTCATAACCCACTTGTTTGTTTTGGCTTTTTGAGATA
AGATCTCTTTCTGTAGTTCAGTGAGACCTGGAATCACCATGCCTCAAACTCATGGCAATCCTCCTGC
CTCAGCTCCATGAGTGCTGTGATTACAGGCATGAGTCACCACGCCTGACTTGTAAATAACAGTTTTC
CCAATTATTTAATTAATATCTGTATCTGCCATCAGACTGGGAGCTCTGCGAAGACAGACAGTAGTT
CTGCCCCAGGGTCGCCAGTCCCCGAGCGAAGGCAGGCAGGCGGGCACACCGCAAAAGCTCAGGCC
CCTAGCAGACCCTGAATGTCCCTGTCTCTCTTTGAACCCTGGTACTTCCTCTGCCTCTGAAACCCAC
ACATACTTTTATCCAGGGGACTGGAGGGAACCTCTACTCCCGCCCTTTCCCTTCTTGGCAAAGGCAC
AGGGCAGGGGCCGCTCTCCCTGTGGGTGACGCACCTAGAGGCGGGCCCGCCCCACAGCGGACGCT
GAGTTGGCCTGGTTGGGCAAGGCCAGGGTTGCCCCGGGGTAGGTTACTCATCTAAGGCTCAGGTAA
GAGGGCCCGGGTTGGAAGGTGGCACACCCAGGGGGGACTCGGAGAGAGCAGGAC
ACAGCT**ATG**

HRE1: -674 bp: **bold violet**; HRE2: -1184 bp: **bold green**; HRE3: - 1558 bp: **bold red**; start codon: **bold**.

2.1.11.2. Primers for HRE mutating CGTG to CTGT

HRE 1 for: 5´-CTTCTCAATTTCAGCTCGACTGTTAGCCCTTTATCCG-3´

HRE 2 for: 5´-ATGGGGTGGAGGGACCACTCTGTGTTTTATTACTCAAT-3´

HRE 3 for: 5´-GTTCTGTCTTGGCCTCCTACTGTCTGAACTACAGGGA-3´

2.1.11.3. Other primers

C4.4A for: 5´-CTACAGCTG CGTGCAAAAGG-3´

C4.4A rev: 5´-GTTGAGTTTGGCGTTGCAT-3´

β-actin for: 5´-TCATGAAGTGTGACGTTGACATCCGT-3´

β-actin rev: 5´-CCTAGAAGCATTTGCGGTGCACGATG-3´

2.1.11.4. Sequence of siRNA targeting C4.4A

Sequence	**Provider**
C4.4A rat, SI02011919	QIAGEN, Hilden
C4.4A rat, SI02011926	QIAGEN, Hilden

2.1.11.5 Plasmids

Plasmid	**Provider**
pEGFP-C1 Plasmid	(Clontech, Palo Alto)
pcDNA3.1/CAT	(Invitrogen, San Diego)
pBLCAT3 und pBLCAT2	(Luckow and Schütz, 1987)
pSUPERneo+gfp	(Oligoengine, Seattle, USA)
p(HA)HIF1α (401Δ603)	(Huang *et al*., 1998)
p(HA)HIF1α	(Huang *et al*., 1998)

2.1.12 Bacterial strain

E.coli DH5α	Genotype: F-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk-,mk+), *pho*A, *sup*E44, *thi*-1, *gyr*A96, *rel* A1, λ- (Invitrogen, Darmstadt, Karlsruhe)
E.coli oneShot®TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG (Invitrogen, Darmstadt)

2.1.13 Computer software

Promoter analysis: Alibaba 2.1: Phttp://darwin.nmsu.edu/~molb470/fall2003/Projects/solorz/aliBaba_2_1.htm: Promoter analysis

Primer design: IDT®: http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer

Nucleotides downloading: http://www.ncbi.nlm.nih.gov/nuccore/NM_021759

FACS analysis: Cell Quest analysis program

Fluorescence analysis: SPOT2.1.2

Signal quantification: ImageJ

Nucleotides alignment: Gentle

2.1.14 Tumor lines

BSp73ASML ($C4.4A^+$)	metastasizing rat pancreatic adenocarcinoma line (Matzku *et al.,* 1983)
BSp73AS ($C4.4A^-$)	non-metastasizing rat pancreatic adenocarcinoma line (Matzku *et. al*, 1983)
BSp73AS1B ($C4.4A^+$)	C4.4A cDNA transfected AS clone (Rösel *et al.,* 1998)
Progressor ($C4.4A^+$)	rat colon carcinoma line (Martin *et al.,* 1983)
804G	rat bladder carcinoma line, LN5 secreting (Homma *et al.,* 1985)
Human A431	human epidermoid line, LN5 secreting (Panneerselvam *et al*., 1985)
Capan2	human pancreatic adenocarcinoma line, metastasizing (Takomori *et al.,* 1996)
Colo357	human pancreatic adenocarcinoma line, metastasizing (Morgan *et al.,* 1980)
8.18	human pancreatic adenocarcinoma line, weakly metastasizing (personal observation) (Tumor Bank, German Cancer Research Center, Heidelberg)
BxPC3	human pancreatic adenocarcinoma line non-metastasizing (Tan *et al.,* 1996)

2.1.15 Rat strain

For experiments 8-12 weeks old BDX rats were used. The rats were bred in the animal facility of the University of Heidelberg under pathogen-free conditions and were provided sterile food and water *ad libitum*.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Cloning

2.2.1.1.1 Dephosphorylation

The formation of a phosphodiester bond between adjacent nucleotides can be repressed by removing the 5' phosphates from both ends of the linear DNA with calf intestinal phosphatase (CIAP, MBI Fermentas). 1-5 µg of digested DNA were mixed with 1 µl of CIAP (5U) and incubated at 37°C for 1 hour. The reaction product was used for ligation.

2.2.1.1.2 Ligation

Joining linear DNA fragments together with covalent bonds is called ligation. Briefly, 1-5 µg of digested and dephosphorylated plasmid DNA were mixed with the insert (to a ratio depending on the amount of both DNA), the T4 DNA ligase and the ligase buffer. The mixture was incubated at 16°C overnight. Ligation products were used for bacteria transformation.

2.2.1.2 Competent bacteria

Competent bacteria are adapted to receive heterogeneous DNA with high efficiency. For experiments, competent bacteria were made chemically. 1ml from bacteria overnight culture was added to 100 ml Luria Bertani (LB) medium without antibiotic and grew till the O.D.600nm reaches 0.5 – 0.6. Cells were spun down in the Sorvall GSA rotor at 5,000 RPM for 5 min at 4^{o}C and bacteria pellet was gently resuspended in 15 ml of filtered transforming buffer I. After 10

min incubation on ice, cells were again spun at 4,000 rpm in the Sorvall GSA rotor for 10 min and the bacterial pellet was resuspended in 2 ml of ice cold transforming buffer II. 100 µl of bacteria were aliquoted in 1.5 ml Eppendorf tubes, shock frozen and stored at -80^{o}C.

2.2.1.3 Transformation

In molecular biology, the transformation is the introduction of a foreign plasmid into competent bacteria to amplify the plasmid. 50 µl of competent bacteria were unfrozen on ice, 0.1 – 1 µg of plasmid was added and kept on ice for 30 min. Then, the cells were heated (heat shock) at 42°C for 45 – 60 seconds and kept on ice for 2 min. 200 µl SOC medium without antibiotics were added and kept on the shaker at 250 rpm for 1hour at 37°C. 10 – 50 µl of the broth were plated on LB agar plate containing selection drug (ampicilline at 100µg/ml). Plates were incubated overnight at 37°C to get colonies.

2.2.1.4 DNA extraction

2.2.1.4.1Miniprep

Single colonies were picked from LB agar plate and put in 3 ml LB medium containing antibiotic, and shaked at 250 rpm at 37°C overnight. 1.5 ml of bacterial culture was used for mini-prep according to the supplier´s recommendations (Miniprep kit, Qiagen). Positive clones were checked on agarose gel with ethidium bromide staining after enzymatic digestion and cultivated at 37°C overnight with shaking for midi-preparation.

2.2.1.4.2 Midiprep

Midiprep System is designed to isolate high-quality and large amount of plasmid DNA for use in eukaryotic transfection. Overnight culture of positive clones was used for midiprep according to the supplier´s recommendations (Midiprep kit, Qiagen).

2.2.1.5 RNA isolation

For total RNA extraction, 8 x 10^6 cells were mixed with 1 ml reagent Tritic YG and incubated at room temperature (RT) for 10 min. Then, 200 µl of chloroform was added and vigourously vortexed for 2- 15 min at RT. After 15 min centrifugation at 12000 RPM at 4°C, the upper aqueous layer was collected and mixed with 500 µl isopropanol. The mixture was centrifuged at 12000 RPM for 10 min at 4°C. The pellet was washed two times with chilled 70% ethanol at 12000 RPM for 5 min at 4°C. Finally, the pellet was dried, re-suspended in 25 ml sterile water and store at -80°C.

2.2.2 Cell biology

2.2.2.1 Cell culture

Tumor cells were cultured in an incubator at 37°C, 5% CO_2, 95% humidity. Cells were maintained in RPMI 1640- or Iscove´s medium, containing 10% heat inactivated fetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin. Where indicated, cells were cultured in the presence of 200µM $CoCl_2$ for 12h, to mimic hypoxic conditions. Confluent cells were detached with trypsin, EDTA or pippeting up and down. The cells were split at a ratio of 1 : 4. Long term cell storage was done by washing the cells once with fresh medium, resuspending the cells in ice-cold FCS/10% DMSO, keeping the cells in vials overnight at -80°C and transferring the vials into liquid nitrogen.

To unfreeze cells from liquid nitrogen, vials were kept in the water bath at 37°C, till a little bit of ice remains, and immediately transferred into 15 ml Falcon tubes containing fresh medium. The cells were centrifuged at 1600 rpm for 4 min. The supernatant was removed and the cells resuspended in fresh medium and transferred into new flask. The cell viability was assessed under the light microscope by trypan blue staining.

2.2.2.2 Transfection of tumor lines

The introduction of nucleic acids into eukaryotic cells by nonviral methods is defined as transfection. Briefly, 12 hours before transfection, 4 x 10^5 ASML cells were seeded in a 6 well-plate. At 70-80% confluency, cells were transfected with 3µg pBLCAT3-C4.4A, 1µg EGFP-C1 or 1.75µg pBLCAT3-C4.4A, 1.25µg p(HA) HIF1α 401Δ603 (HIF-1α with oxygen-dependent degradation domain deletion), 1µg EGFP-C1 using Lipofectamine™ 2000 according to the supplier´s recommendations.

To generate a stable C4.4A knock down line, 48 hours after transfection, C4.4A siRNA transfected cells were put under selection pressure with neomycin. Transfected cells were selected by cloning under limiting dilution with 1 or 3 cells/well in 96 well-plates. The cells were checked by FACS after each recloning step until uniformly positive clones were obtained.

2.2.2.3 Chloramphenicol acetyl transferase (CAT) assay

After transfection with the *C4.4A* promoter constructs and pEGFP-C1 as described above, cells were washed two times with PBS. After 5 min incubation (4°C, 1 ml TEN buffer), cells were scrapped, transferred to Eppendorf tubes and centrifuged (5 min, 5000 rpm). Cells were resuspended in 80 µl ice cold Tris/Cl, and lysed by freezing / thawing. Lysates (900 µl) were centrifuged at full speed for 5 min. The supernatant was transferred into Eppendorf tubes. After determining the protein content by Bradford, samples were kept at -80°C.

To normalize the CAT extracts, remaining 100 µl of cell suspension were used to determine via FACS the transfection efficiency based on GFP expression. Using the protein concentration and the transfection efficiency, the volume of protein for the CAT reaction was determined as followed: Volume = 10% x µl volume of lysate (containing 100µg)/transfection efficiency. The CAT reaction was prepared by mixing: 2 µl ^{14}C labeled Chloramphenicol, 20 µl 4 mM Acetyl CoA (dissolved in 0.25 M Tris), x µl 0.25 M Tris/Cl, pH7.5, x µl cytoplasmic

cell extract, for a total volume of 150 µl. The mixture was incubated at 37 °C for 3 h. Eight hundred µl of ethylacetate was added, then vortexed. After centrifugation (13000 rpm, 4°C, 5 min), the supernatant was transferred to Eppendorf tubes and evaporated in a Speedvac. The chloramphenicol pellet was resuspended in 12 µl ethyl acetate for thin layer chromatography (TLC). Prior to loading the TLC sheet with ^{14}C chloramphenicol-ethylacetate, the TLC chamber was equilibrated with 150 ml mixture chloroform/methanol (19:1 v/v) and 2 pieces of Whatmann paper. The probes were separated for 45 min. The dried TLC sheet was exposed to x-ray film for 12 h. The spots were quantified using the Phosphor Imager System.

2.2.2.4 Adhesion assay

Adhesion of cells treated with or without $CoCl_2$ to BSA- and LN5-coated 96-well plates was determined after 30 min and 240 min at 37^{o}C. Non-adherent cells were removed by washing. Adherent cells were fixed with ice cold methanol and stained with 0.1% crystal-violet for 10 min. After washing with distilled water, drying and dissolving in 10% acetic acid, the OD at 595 nm was measured.

2.2.2.5 Migration assay

Migration was evaluated in Boyden chambers seeding cells in the upper chamber (RPMI/1%BSA) with/without $CoCl_2$ and/or protease inhibitors. The lower chamber separated by an 8µm pore size polycarbonate-membrane, contained RPMI/1%BSA or 804G supernatant. Migrated cells on the lower surface of the membrane were fixed with ice cold methanol and stained with 0.1% crystal-violet for 10 min. After washing with distilled water, drying and dissolving in 10% acetic acid, the OD at 595 nm was measured.

For *in vitro* wound healing, a subconfluent monolayer was scratched with a pipette tip. Wound closure, followed after 0h-72h by light microscopy, is presented as percentage of reduction of the freshly wounded area.

2.2.2.6 Apoptosis assay

To distinguish between necrotic and apoptotic cells, the AnnexinV-APC and propidium iodide (PI) (R & D systems, Wiesbaden-Nordenstadt, Germany) double staining was used. DNA fragmentation and the alteration of the permeability and phospholipid composition of the plasma membrane are the two main events of the apoptotic process. During apoptosis, phosphatidylserine translocates from the cytoplasmic side of the membrane to the extracellular side, and can be detected with AnnexinV. Propidium iodide (PI) dye is known to bind to the DNA of leaky cells. These features are exploited in apoptosis assay. Early apoptotic cells bind only to annexin V, late apoptotic cells bind to annexin V and Propidium iodide and necrotic cells bind to Propidium iodide. Cells were seeded in 96 well plate and treated with or without cis-platin, in the presence or absence of 200µM $CoCl_2$. After 24h, 48h and 72h incubation, the cells were washed twice with the binding buffer by centrifugation (1600 rpm, 4 min) and stained with a mixture of AnnexinV-APC – PI for 15min in the dark at room temperature. The apoptotic status of cells was assessed by FACS.

2.2.2.7 Soft agar assay

Tumor cells in 0.3% agar were seeded on a preformed 1% agar layer and kept in the incubator. Where indicated, the cells were treated with 0.5 or 1µg/ml cisplatin. After 3 weeks, the colonies were counted and the pictures taken.

2.2.2.8 Immunofluorescence

Cells seeded overnight on BSA-, LN1-, LN5- or FN-coated cover slides were fixed, permeabilized, blocked, incubated with primary antibody (60min, 4^{o}C),

fluorochrome-conjugated secondary antibody (60min, 4°C), blocked, incubated with a second, dye-labeled primary antibody (60min, 4°C) and washed. Where indicated, cells were removed by EDTA. Cover slides were mounted in Elvanol. Shock frozen skin sections (7µm) were exposed to primary antibody, biotinylated secondary antibody and alkaline phosphatase-conjugated avidin-biotin complex solutions. Sections were counter stained with H&E. Digitized images were generated using a Leica DMRBE microscope, a SPOT CCD camera and Software SPOT2.1.2.

2.2.2.9 Flow cytometry

The flow cytometry is a technique used to measure , besides other fluorescence labelling of individual cells. Briefly, 1-3 10^5 cells detached with EDTA, were transferred to round bottomed 96 well plates. Cells were washed twice with FACS washing buffer (1xPBS, 1% FCS, pH7.4) and incubated with 40µl of the primary antibody (1-5µg/ml) for 30min at 4˚C. Then, cells were washed again twice, and incubated with 40µl of the secondary, fluorochrome-conjugated antibody (0.3-0.5µg/ml) for 30min at 4˚C in the dark. For intracellular staining, cells were fixed in 1% formalin (20min, 4˚C), washed and permeabilized with 0.2% Tween in FACS washing buffer before incubation with the primary and secondary antibodies.

For CAT assay standardization, cells were co-transfected with the EGFP-C1 plasmid to evaluate transfection efficacy through EGFP expression.

Cells were analyzed in a FACScan using the Cell Quest analysis program (BD, Heidelberg, Germany).

2.2.2.10 Vesicle depletion and exosome preparation

Cells were cultured for 48h in serum-free medium. Cells were treated with or without 200 µM $CoCl_2$ for 12 hours. Supernatants were cleared by stepwise centrifugation (2x10min, 500g, 1x20min, 2000g, 1x30min, 10000g), then,

followed by 2.5h of ultracentrifugation at 100,000g using SW41Ti rotor in Beckman Coulter ultracentrifuge. The exosomes are recovered in the pellets, which were re-suspended in 1x PBS and the concentration measured by Bradford assay. The resuspended exosomes were stored at -80˚C.

2.2.3 Protein Biochemistry

2.2.3.1 Biotinylation

Biotinylation is the process of attaching biotin to proteins and other macromolecules. The biotin-avidin/-streptavidin interaction is commonly exploited to detect and/or purify proteins because of the high affinity between these molecules. Briefly, cells were washed in cold HEPES buffer without detergent at 1600 RPM for 4 min at 4°C. After washing, cells were incubated with 0.1mg/ml biotin-X-NHS in HEPES buffer for 30 min at 4°C with shaking. Cells were quenched with PBS-200mM Glycine and lysed in HEPES buffer (containing 1% Brij96, 1mM PMSF and 1x protease inhibitor cocktail) for 60min at 4°C. Exosomes biotinylation followed the same procedure as above, after quenching and washing, exosomes were pelleted by ultracentrifugation at 100,000g for 2,5h.

2.2.3.2 Immunoprecipitation (IP)

Cells were lysed in HEPES buffer (containing 1% Brij96, protease inhibitor cocktail) for 60min at 4°C with shaking and centrifuged at 13,000 rpm for 10 min at 4°C. 1 mg of cell lysates or 100 µg of exosomal lysates were incubated with antibody (2µg/ml or 200µl hybridoma supernatant) overnight and precipitated with 5% ProteinG Sepharose for 1h at 4°C. Washed complexes were dissolved in Laemmli buffer, boiled at 95°C for 5min and centrifuged shortly to separate sepharose beads from proteins. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes (30V, 12h, 4°C). Membranes were blocked, blotted with primary and HRP-conjugated secondary

antibodies (1h, RT) and developed with the ECL kit or were stained with Coomassie blue.

2.2.3.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins according to size. The SDS is a detergent that can dissolve hydrophobic molecules but also has a negative charge attached to it. The PAGE allows different sized proteins to move at different rates. Two gels were used to separate proteins. At the top, the stacking gel (375mM Tris pH6.8, 0.1%SDS, 4% acrylamide-bisacrylamide, 0.1%TEMED (v/v), 0.1% (w/v) ammonium persulphate) concentrates proteins and allows them to enter the resolving gel at the same time. At the bottom, the resolving gel (375mM Tris pH8.8, 0.1%SDS, 10-12% acrylamide-bisacrylamide, 0.1%TEMED (v/v), 0.1% (w/v) ammonium persulphate) allows the separation of proteins. Once the gel has completely polymerized, boiled samples, under non-reduced or reduced conditions (with beta-mercaptoethanol) were loaded and the gel was run in running buffer at 60 volts. After electrophoresis, gels were either subjected to coomassie blue staining or western blot.

2.2.3.4 Western blotting

Western blot allows the detection of specific protein and the measurement of relative amounts of the protein present in different samples. The proteins were transfered to nitrocellulose membranes using pre-wet materials sacked as followed: case (clear side), sponge, Whatman paper, membrane, gel, Whatman paper, sponge and case (black side). This set was placed in the transfer apparatus with black side facing black. The transfer was performed at 30V, overnight, at 4°C. The transfer was quickly checked by staining the membrane with 1x Ponceau S for a minute and destaining with distilled water. The membrane was blocked for 1 hour in 10 ml 1x PBS + 5% non-fat dry milk + 0.1%Tween 20 (PBST), with shaking. The blocking is followed by blotting with the primary

antibody, overnight at 4°C on a shaking platform. After three times washing with 1x PBS + 0.1%Tween 20, the membrane was probed with horse radish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10000 in PBST) for 1h at room temperature, then, washed three times. For biotinylated proteins, the membrane was incubated with Streptavidin-peroxidase. Enhanced Chemiluminescence system (ECL, Amersham Biosciences) and X-ray film (Amersham Biosciences) were used to detect the proteins.

2.2.3.5 Coomassie staining

After electrophoresis, the gel was covered with the coomassie brilliant blue solution for 30 min on a shaker. After staining, the gel was destained with the coomassie destaining solution (50% (v/v) methanol and 10% (v/v) acetic acid in distilled water) until the protein bands were clearly visible.

2.2.4 Animal experiments

A 1cm diameter full thickness skin area was excised from the shaved back of 8 weeks old BDX rats. At the time of excision, after 4days and 7days, rats received 100µg control IgG or C4.4 in 100µl PBS, perilesionally. Sterile gauze covering the wound was fixed with a whole body bandage. A 2cm diameter area, including the wound, excised immediately, after 1day, 4days, 7days and 10days, was shock frozen. Rats received tumor cells ($1x10^6$ or $5x10^6$) intrafootpad (ifp) or intraperitoneally (ip). PBS or cisplatin (1µg/g body weight) were given ip after 2d and 23d. Rats were controlled weekly for local, draining lymph node or intraperitoneal tumor growth, ascites, short breathing or weight loss. Animals were sacrificed when the draining lymph node (LN) reached a mean diameter of 2cm, ascites became obvious, rats became pale, fatigue, lost >10% weight or latest after 120d. Animal experiments were Government-approved (Baden-Wuerttemberg).

2.2.5 Statistical analysis

For all assays, values represent the mean (±SD) of triplicates and/or 3 repetitions. P-values (Student's t-test, *in vitro* assay, *in vivo* Kruskal-Wallis assay) <0.05 were considered statistically significant.

3. RESULTS

C4.4A has been described as a metastasis associated molecule that also is involved in wound repair. Its function remains elusive. As tumor progression and wound repair are frequently associated with a state of hypoxia, I aimed to explore whether expression and/or functional activity of C4.4A are regulated by hypoxia. I started with exploring the question whether C4.4A transcription is regulated by hypoxia. It has been previously described by Fries and coworkers (2007) that transcription of C4.4A requires the transcription factor C/EBPβ. Moreover, transcription is further increased in the presence of Jun-D and c-Jun. Furthermore, uPAR, a structural homolog of C4.4A also was reported to become regulated under oxygen stress. Finally, the C4.4A promoter contains some putative HIF responsive elements (HRE). Thus, hypoxia might well regulate C4.4A expression.

3.1 Hypoxia-induced C4.4A up-regulation

To evaluate the impact of hypoxia on C4.4A expression, ASML and Prog (two metastatic C4.4A$^+$ rat tumor lines), AS1B1 cells (C4.4A cDNA transfected AS cells) (Matzku *et al.,* 1983; Rösel *et al.*, 1998) and human pancreatic tumor lines (Colo357, 8.18, Capan2, BxPC3) were used.

Cells were cultured in the presence of $CoCl_2$, to mimic hypoxic conditions (Maytin *et al.,* 1999). High C4.4A expression in ASML and Prog cells was increased already after 6h 200µM $CoCl_2$-treatment and increased further during a 12h culture period. AS1B1 cells showed a minor increase only after 12h of culture in the presence of $CoCl_2$. AS cells remained C4.4A$^-$ (Fig.5A and 5B). Two human pancreatic cancer cell lines, Colo357 and Capan2, which express C4.4A at a medium to low level, responded to $CoCl_2$-treatment with a dose- and time-dependent increase in C4.4A expression, where a subfraction showed very high expression after 200µM $CoCl_2$-treatment. 8.18 cells, which do not express

C4.4A revealed very weak expression in about 15% of cells after 12h 200µM $CoCl_2$-treatment. BxPC3 cells remained C4.4A$^-$ (Fig.5C and 5D).

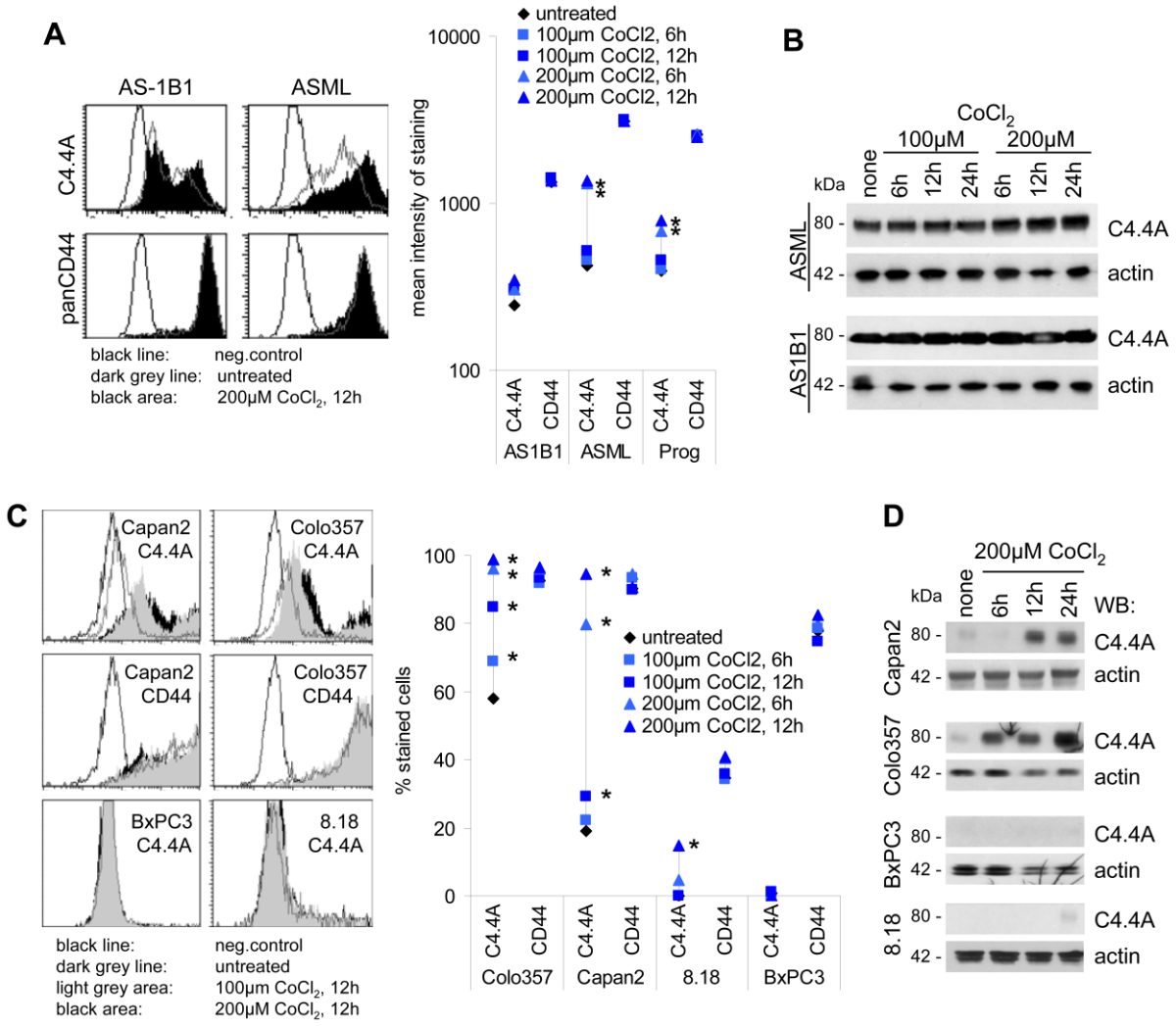


Figure 5: Hypoxia-induced C4.4 up-regulation. (A,B) ASML, Prog and AS1B1 cells and **(C,D)** the human pancreatic cancer lines Capan2, Colo357, BxPC3 and 8.18 were cultured in the presence of titrated amounts of $CoCl_2$ for 6h-24h. **(A,C)** Expression of C4.4A was evaluated by flow cytometry. Representative examples and **(A)** mean values of intensity of C4.4A expression or **(C)** the mean percentage of stained cells in 3 different experiments is shown. CD44 expression served as control. Significant differences due to $CoCl_2$-treatment: *. **(B,D)** Lysates of untreated and $CoCl_2$-treated cells were separated by SDS-PAGE, transferred and blotted with anti-C4.4A. Actin served as control.

As shown by Western Blot and Flow Cytometry, C4.4A expression increases under hypoxia in rat (ASML, Prog and AS1B1) and human (Capan2, Colo357, BxPC3 and 8.18) tumor lines. C4.4A expression is not or very weakly induced by hypoxia in cell lines that do not express C4.4A in normoxia. Thus, hypoxia

contributes to C4.4A up-regulation, but may not suffice to induce *C4.4A* transcription.

3.2 *C4.4A* transcription is not promoted by HIF1α

Under hypoxic conditions, HIF1α is stabilized and accumulates. Thus, it translocates into the nucleus and interacts with some co-activators, such as P300/CBP, to drive the expression of target genes. Under normoxic conditions, HIF1α is subjected to proteosomal degradation. AS, AS1B1 and ASML cells express HIF1α at low level under normoxia. In ASML cells, HIF1α showed a strong, dose-dependent increase after 12h $CoCl_2$-treatment. Up-regulation was weak after 6h and declined beyond 12h. An increase in HIF1α was also seen in $CoCl_2$-treated Capan2 and BxPC3 cells. As the latter do not express C4.4A after $CoCl_2$-treatment, this finding suggests HIF1α by itself to be insufficient to induce *C4.4A* transcription (Fig.6).

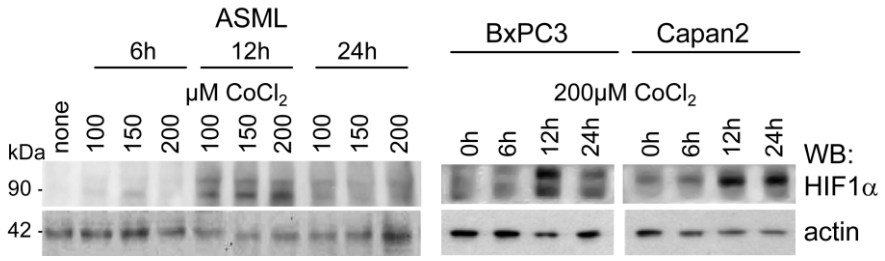
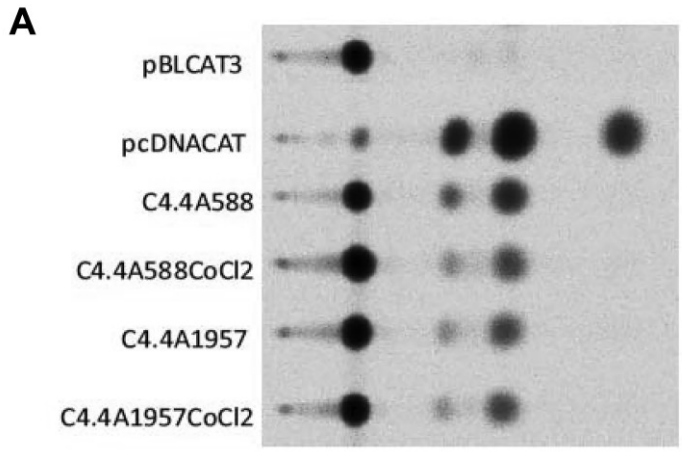


Figure 6: HIF1α expression. ASML, Capan2 and BxPC3 cells were cultured in the presence of 200µM $CoCl_2$ for 6h-24h. HIF1α expression was evaluated by WB, actin served as control.

To study the *C4.4A* promoter activity, I performed the Chloramphenicol Acetyl Transferase (CAT) assay. CAT is a bacterial enzyme which inactivates chloramphenicol by acetylating it. In eukaryotic cells, there is no background CAT activity. Thus, the CAT assay is suitable to examine the promoter activity in the rat pancreatic adenocarcinoma line. The *C4.4A* promoter contains 3HRE at -674 bp, -1184 bp and -1558 bp (see 2.1.11.1) and we controlled whether HIF1α acts as a co-transcription factor in hypoxia. The *C4.4A* promoter from -588 or from -1957 to -1bp were inserted into promoterless pBLCAT3 vector.

After transfection of ASML cells with *C4.4A* promoter constructs, promoterless pBLCAT3 vector (negative control), pcDNA3.1/CAT (positive control) and pEGFP-C1 for standardization, transfected cells were treated with 200µM $CoCl_2$ for 12 h. Then, cells were lysed and lysates were used for the CAT reaction. Finally, probes were subjected to thin layer chromatography (TLC) and developed using the affinity of ^{14}C-labeled chloramphenicol. The CAT activity quantified by the Phosphor Imager System showed no significant changes (Fig.7A, 7B). Furthermore, to ensure that HIF1α does not directly influence the promoter activity, I mutated the 3 HRE using the primers listed in 2.1.11.2. After confirmation of the right mutation by sequencing, ASML cells were transfected with the 3 mutated HRE. Once more, we observed that the CAT activity remained unaltered in normoxia or hypoxia (Fig.7C) and only a slight increase in CAT activity was observed upon co-transfection with pCDNA3-HIF1α, irrespective of HRE mutations (Fig.7D).

The observation that the CAT activity does not vary when cells were transfected with non mutated or mutated constructs, or cotransfected with HIF1α expressing vector, led us to emphasize that hypoxia-induced C4.4A up-regulation does, at least, not predominantly proceed at the transcription level.



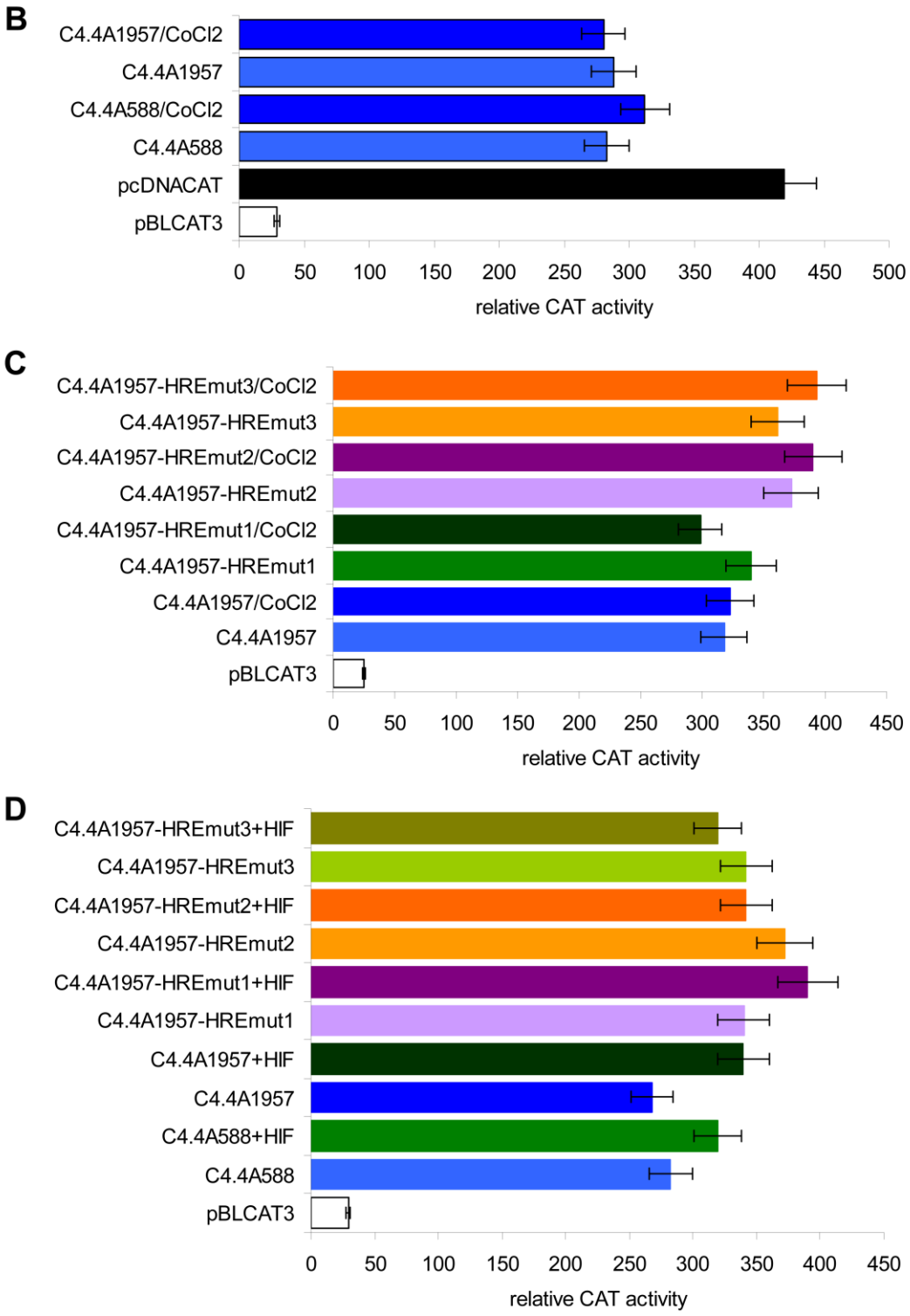


Figure 7: HIF1α does not promote *C4.4A* transcription. (A-D) The -1 to -1957bp C4.4A promoter region with/without the 3 HRE mutations and the -1 to -588bp sequence (no HRE) were cloned into the pBLCAT3 vector. The pcDNA3.1CAT vector (CMV promoter) served as positive, the promoterless pBLCAT3 vector as negative control. ASML cells were co-transfected with the EGFP-C1 plasmid for standardization. Cells were cultured in the presence or absence of 200µM $CoCl_2$ for 12h. **(A,B)** Impact of $CoCl_2$; **(C)** impact of the 3 HRE (mutated); **(D)** impact of HIF1α as revealed by co-transfection. **(A-D)** CAT activity was quantified using a PhosphorImager system. Relative CAT activity (mean±SD of 3 experiments) is shown. No significant differences have been seen.

Up-regulated C4.4A expression under hypoxia apparently does not rely on HIF1α -induced *C4.4A* transcription. Nonetheless, hypoxia promoted C4.4A up-regulation. To exclude an only *in vitro* phenomenon, I next evaluated C4.4A expression in wound repair.

3.3 Hypoxia-induced C4.4A up-regulation in wound repair

Keratinocytes express C4.4A and several studies report that C4.4A might be engaged in wound repair (Jacobsen *et al.*, 2008; Hansen *et al*., 2004; Paret *et al.,* 2005). To control these suggestions, I evaluated C4.4A expression after skin wounding. To obtain hints towards a functional involvement of C4.4A, rats were concomitantly treated with the C4.4A-specific antibody C4.4. Controlling for C4.4A expression and the impact of C4.4 on wound repair after full thickness skin excision, revealed striking C4.4A up-regulation at the leading front of migrating keratinocytes and wound closure was strongly delayed in C4.4-treated rats (Fig.8).

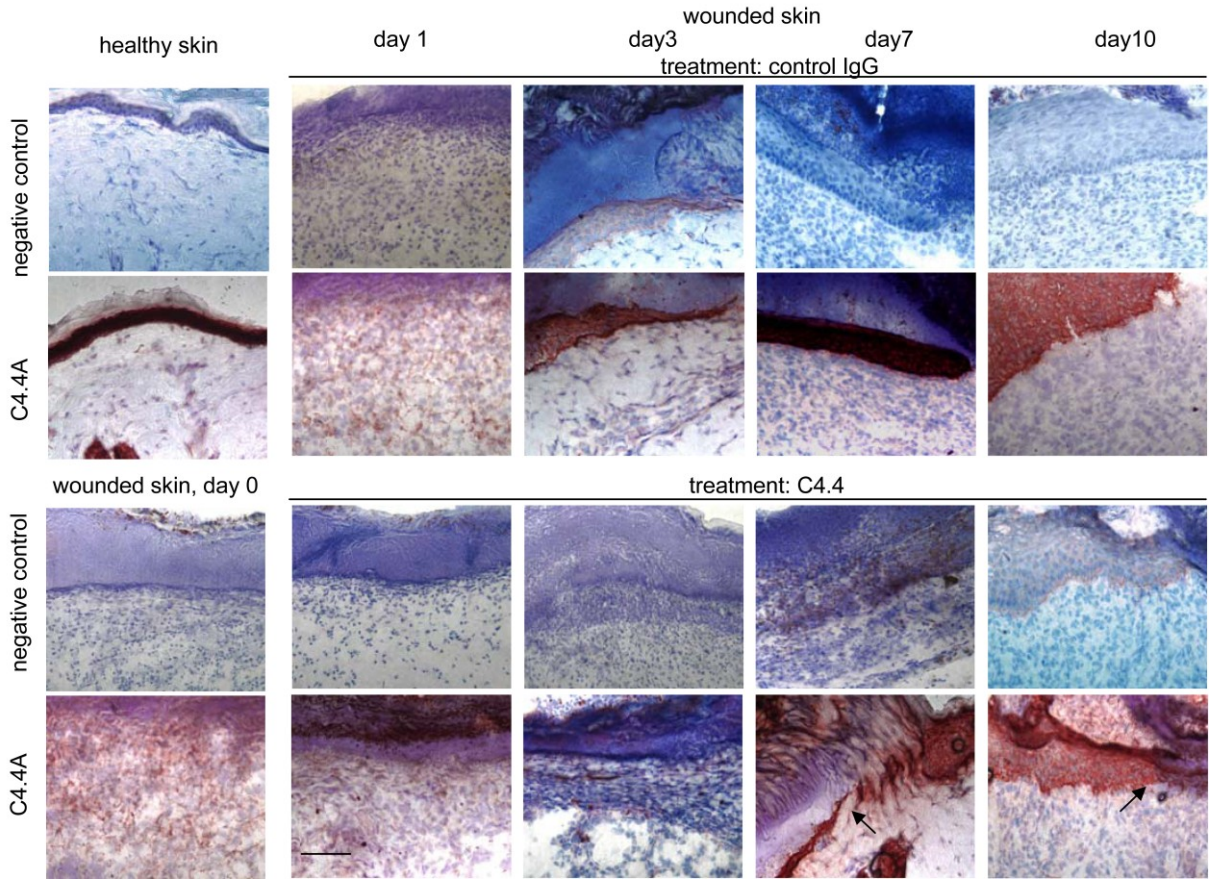


Figure 8: Keratinocyte migration during wound healing and C4.4A expression. The back skin of BDX rats was wounded by a 1cm diameter full thickness skin excision. Rats received at day 0, 4 and 7 after wounding a perilesional injection of 100µg control IgG or C4.4. At the time of wounding, after 1, 4, 7 and 10 days a 2 cm area around the wound was excised and shock frozen. Slices (7µm) were stained with C4.4 or mouse IgG1 (control) and Mayer's hematoxilin (Scale bar: 100µm). Arrows indicate the migration front of keratinocytes.

These findings confirmed upregulated C4.4A expression in hypoxia and, importantly, provided evidence for a functional contribution of C4.4A, as a blocking antibody significantly affects cell migration on the extracellular matrix.

3.4 The engagement of C4.4A in matrix adhesion and migration

The basal lamina is rich in LN5 and C4.4A is a receptor for LN1 and LN5. Thus, I questioned whether the engagement of C4.4A in wound repair may be due to the interaction between C4.4A and LN5. Adhesion to BSA- or LN5-coated plastic was evaluated for ASML ($C4.4A^+$, $\alpha6\beta4^+$), Prog ($C4.4A^+$, $\alpha6\beta4^+$), AS ($C4.4A^-$, $\alpha6\beta4^-$) and AS1B1($C4.4A^+$, $\alpha6\beta4^-$) cells, which all adhered better to LN5 than BSA. In addition, after 30min, significantly more $CoCl_2$-treated than untreated ASML, Prog and AS1B1, but not AS cells adhered to LN5 (Fig.9A). As the increase in binding was more pronounced in ASML and Prog, which both and distinct to AS, express α6β4, we next asked for the contribution of C4.4A versus α6β4 (Rabinovitz *et al*., 1996) to LN5 adhesion. Adhesion of ASML and Prog were both inhibited by B5.5 and C4.4, whereas binding of AS1B1 cells was only inhibited by C4.4. Notably, inhibition by both antibodies was stronger in $CoCl_2$-treated Prog cells (Fig.9B). This was a first indication that under hypoxia C4.4A may possibly cooperate with α6β4.

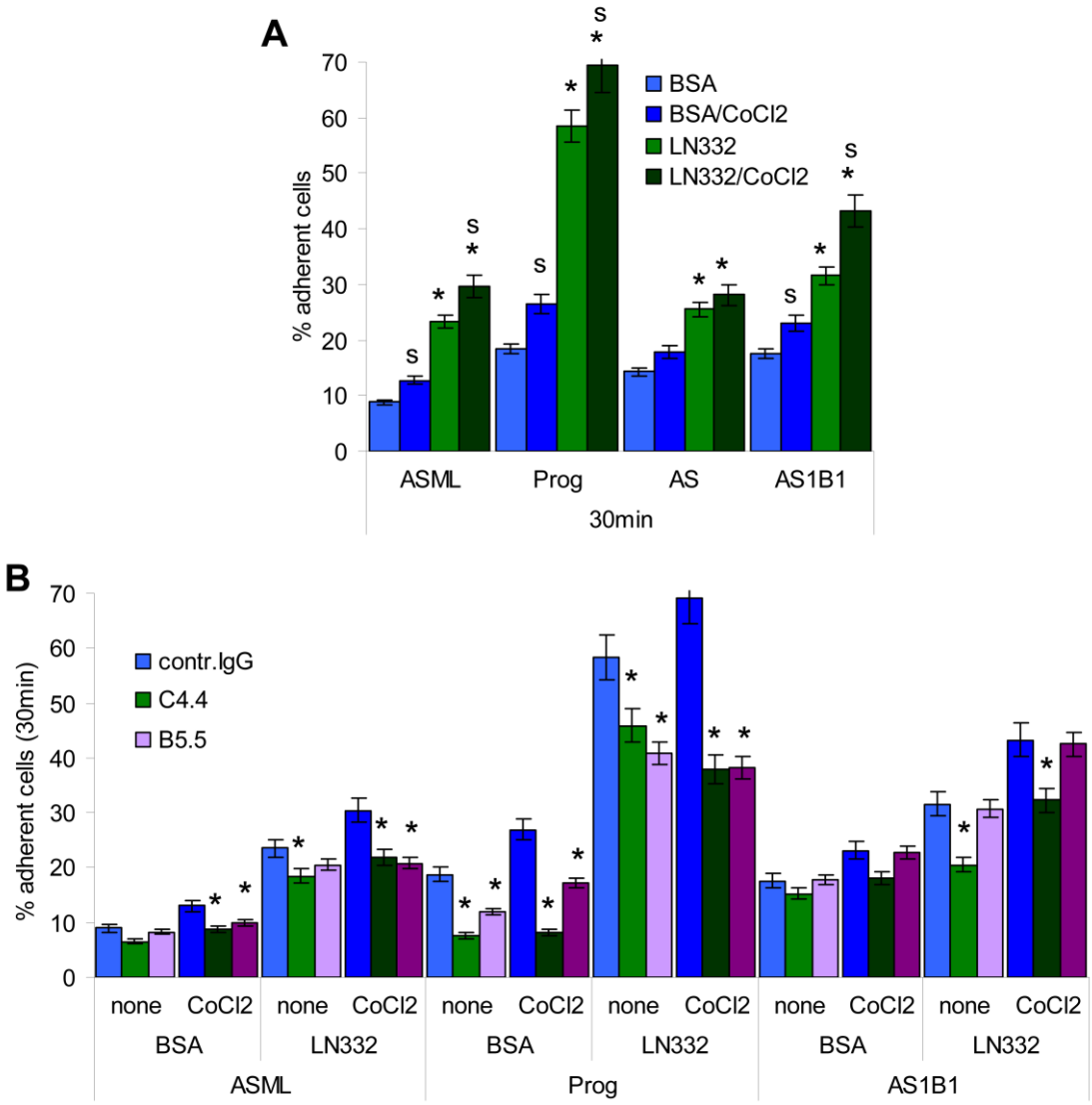


Figure 9: The impact of hypoxia and up-regulated C4.4A expression on LN5 adhesion. (A, B) Untreated and $CoCl_2$-treated ASML, Prog, AS and AS1B1 cells were seeded on BSA- or LN5 (LN332)-coated plates and cultured for 30min in the presence or absence of $CoCl_2$. **(B)** Cultures contained, in addition, C4.4 or B5.5 (10µg/ml). **(A, B)** The percentage of adherent cells (mean±SD of triplicates) is shown. **(A)** Significant differences between BSA and LN5: *; significant difference of untreated versus $CoCl_2$-treated cells: s. **(B)** Significant antibody inhibition: *.

To control whether this cooperativity between C4.4A and α6β4 is supported by proximity or a direct association between C4.4A and α6β4, I first searched for co-localization. C4.4A and α6β4 did not co-localize in ASML and hardly in Prog cells, when seeded on BSA-coated plates. Some co-localization was seen in the presence of $CoCl_2$ or on LN5-coated plates. Instead, C4.4A and α6β4

strongly co-localized in $CoCl_2$-treated ASML and Prog cells seeded on LN5 (Fig.10A).

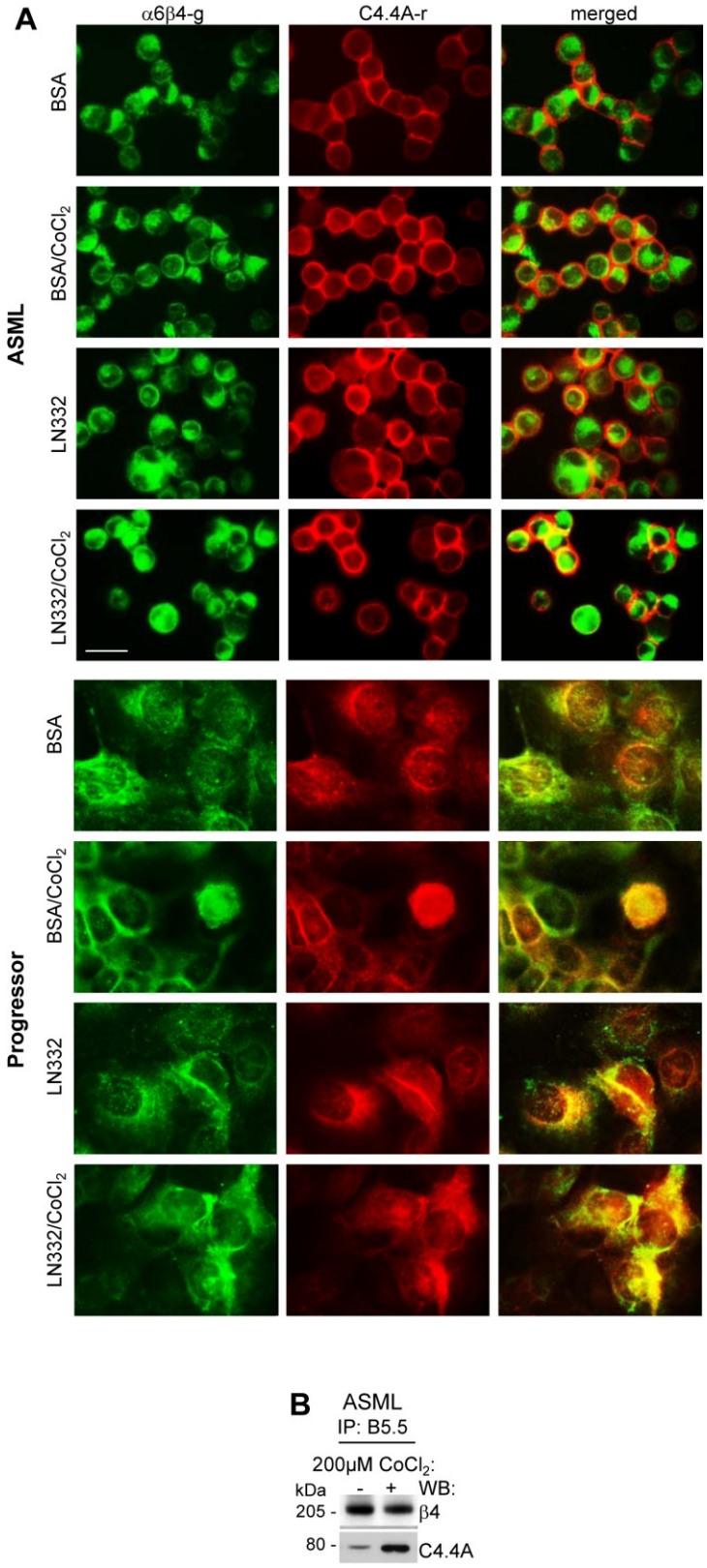


Figure 10: Co-localization of C4.4A with α6β4 under hypoxia. (A) ASML and Prog cells were cultured on LN5 (LN332)-coated cover slides in the absence or presence of $CoCl_2$. Fixed cells were stained with B5.5 / anti-mouse IgG-Cy2 (g) and, after blocking, with C4.4-TxR (r). Single fluorescence staining and digital overlays are shown (Scale bar: 10µm). **(B)** ASML cells were cultured for 16h in the presence or absence of $CoCl_2$. Lysates were immunoprecipitated with B5.5. After SDS-PAGE and protein transfer, membranes were blotted with C4.4 and anti-β4.

Notably, hypoxia not only strengthened co-localization, but also supported a direct association between the two molecules as the amount of C4.4A co-immunoprecipitating with α6β4 was close to 10-fold increased (Fig.10B).

Taken together, hypoxia increases LN5 adhesion, which is equally well inhibited by C4.4 and B5.5 indicating that LN5 adhesion might pursue by a C4.4A-α6β4 complex, which has been confirmed by co-localization and co-immunoprecipitation of the two molecules.

We had reported that C4.4A-mediated LN5 binding is transient and could be restored in the presence of a protease inhibitor (Rösel *et al.,* 1998). This also accounts for $CoCl_2$-treated cells. After 4h, adhesion to LN5 did not consistently exceed adhesion to BSA and the adhesion supporting effect of $CoCl_2$-treatment was weak or abolished. Adhesion of $CoCl_2$-treated cells was partly restored in the presence of a broad serine protease inhibitor (aprotinin) and, more efficiently, a broad MMP inhibitor (MMP-Inh.II) (Fig.11).

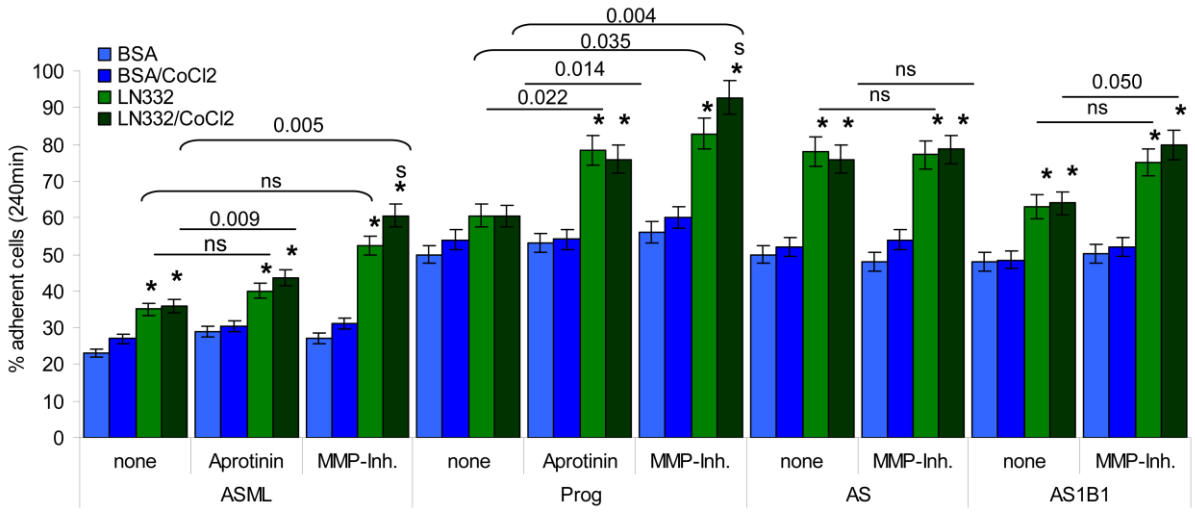
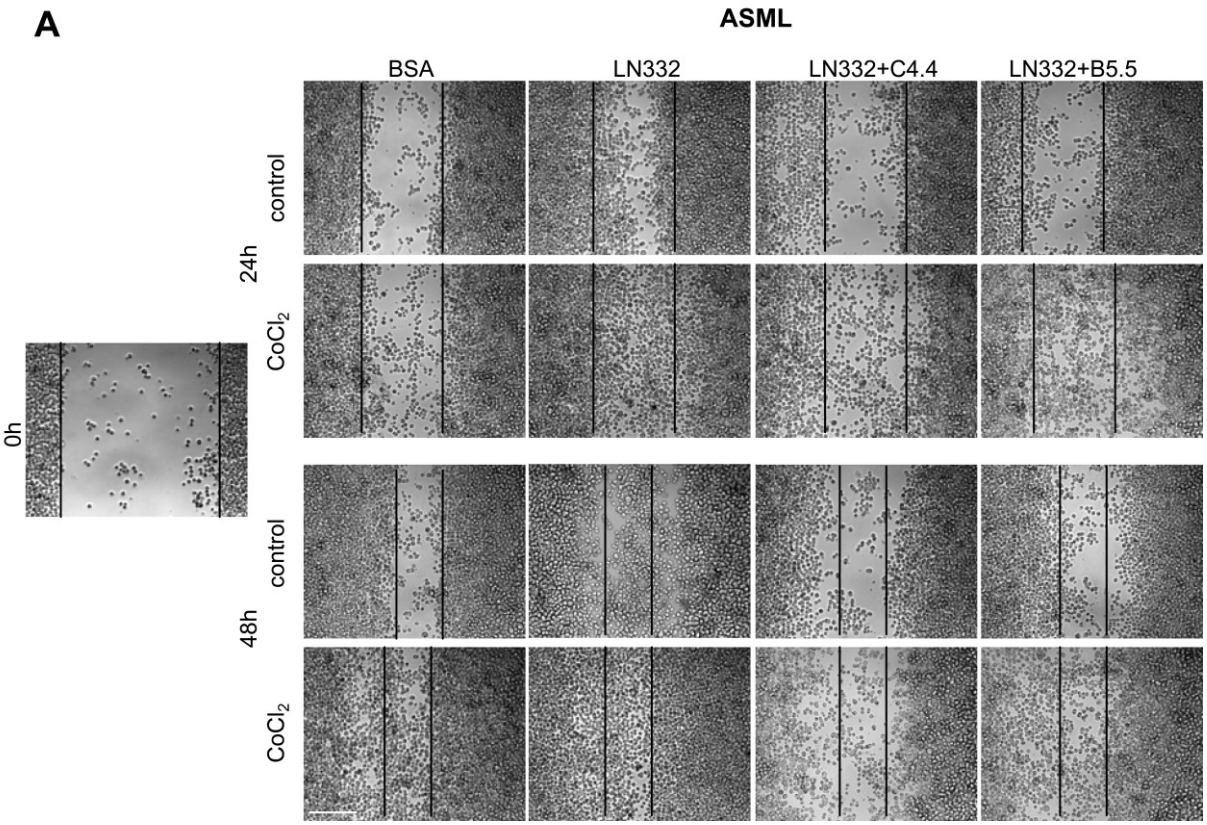
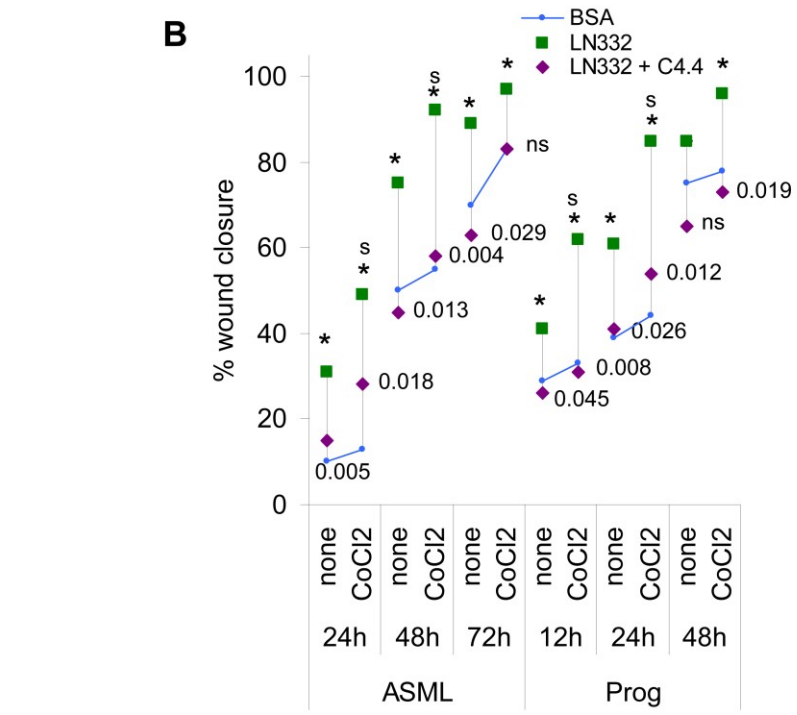


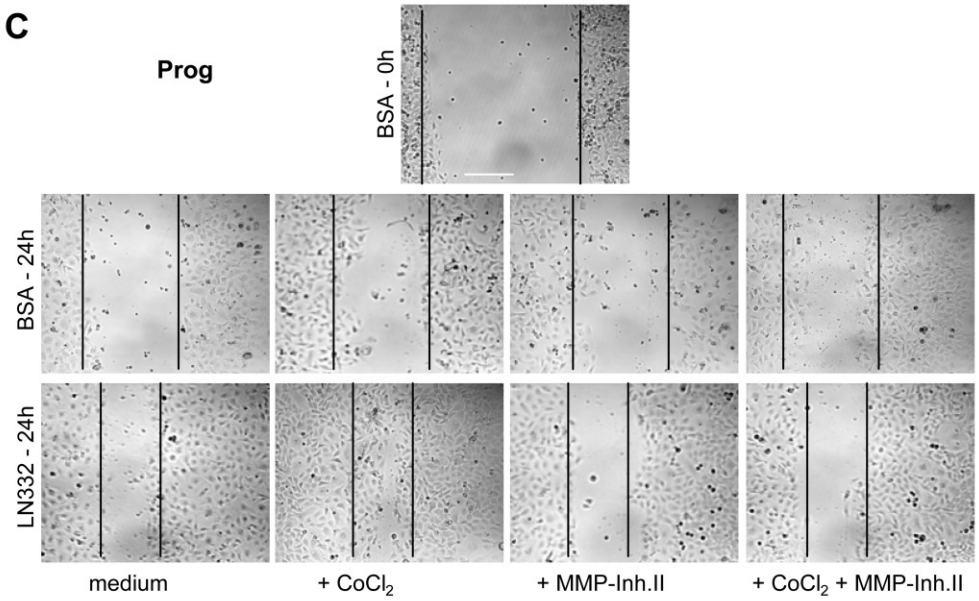
Figure 11: The impact of protease inhibitors on LN5 adhesion: Untreated and $CoCl_2$-treated ASML and Prog cells were seeded on BSA- or LN5 (LN332)-coated plates. Where indicated, cultures

contained $CoCl_2$ and/or aprotinin or MMP-Inh.II. Adhesion was measured after 4h. The percentage of adherent cells (mean±SD of triplicates) is shown. Significant differences between BSA and LN5: *; significant difference of untreated versus $CoCl_2$-treated cells: s; for Aprotinin and MMP-Inh.II inhibition p-values are shown.

While LN5 adhesion decreased with time, hypoxia enhanced migration of ASML and Prog cells on LN5 as revealed by *in vitro* wound healing evaluated 24 h and 48 h after wounding. In addition, both C4.4 and B5.5 inhibited wound closure on LN5-coated plates in the presence or absence of $CoCl_2$ (Fig.12A). In line with the MMP-Inh.II-restored adhesiveness to LN5, MMP-Inh.II interfered with migration. MMP-Inh.II exerted no or a minor effect on wound healing of Prog and ASML cells on BSA, but significantly inhibited wound healing on LN5 in the presence of $CoCl_2$ (Fig.12B).




B
BSA
LN332
LN332 + C4.4
% wound closure
100
80
60
40
20
0
0.005
0.018
0.013
0.004
0.029
ns
0.045
0.008
0.026
0.012
ns
0.019
none
CoCl2
24h
48h
72h
12h
24h
48h
ASML
Prog


C
Prog
BSA - 0h
BSA - 24h
LN332 - 24h
medium
+ CoCl2
+ MMP-Inh.II
+ CoCl2 + MMP-Inh.II

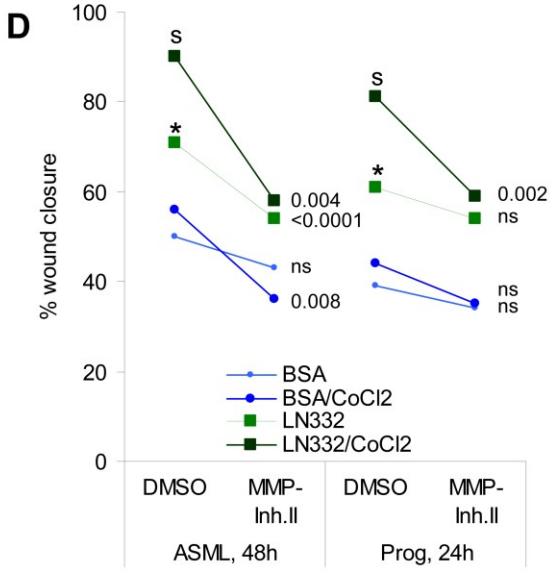

D
% wound closure
100
80
60
40
20
0
0.004
<0.0001
ns
0.008
0.002
ns
ns
ns
BSA
BSA/CoCl2
LN332
LN332/CoCl2
DMSO
MMP-Inh.II
ASML, 48h
Prog, 24h

Figure 12: The impact of C4.4A, α6β4 and a protease inhibitor on wound closure. (A-D) Subconfluent monolayers of ASML and Prog cells on BSA- or LN5 (LN332)-coated plates were scratched and wound closure was controlled in the presence or absence of C4.4 or B5.5 and $CoCl_2$ for 48h-72h. In **(C, D)**, cultures contained MMP-Inh.II. Wound closure was controlled using an inverted microscope. **(A, C)** Representative images (Scale bar: 50µm) and **(B, D)** the mean (triplicates) of wound closure are shown. Significant differences between BSA and LN5: *; significant difference of untreated versus $CoCl_2$-treated cells: s; for antibody and MMP-Inh.II inhibition p-values are shown.

The increased migratory activity induced by hypoxia also accounted for transwell migration of ASML and Prog cells, which was promoted by $CoCl_2$-treatment and inhibited by C4.4 and B5.5 (Fig.13A). Transwell migration towards LN5 was inhibited by MMP-Inh.II, although the effect was weak for Prog cells (Fig.13B).

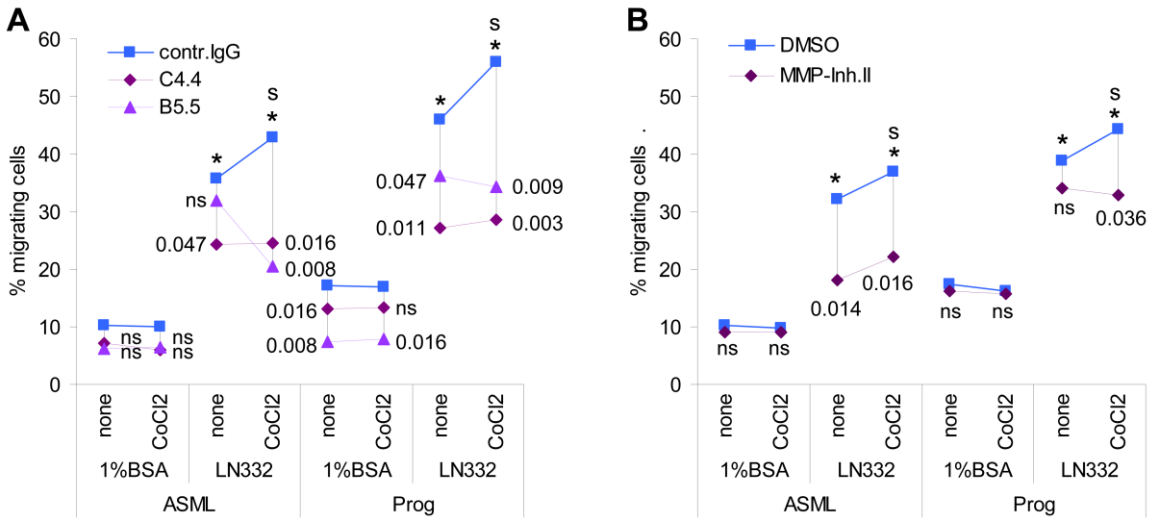


Figure 13: The impact of protease inhibitors on LN5 transwell migration. (A, B) Cells were seeded in Boyden chambers, the lower chamber contained RPMI/1%BSA or 804G supernatant. **(A)** Cells were pre-incubated with C4.4, B5.5 or mouse IgG1. **(B)** Cultures contained MMP-Inh.II. Migration was evaluated after 16h. The mean (triplicates) of transwell migrating cells is shown. Significant differences between BSA and LN5 (LN332): *; significant difference of untreated versus $CoCl_2$-treated cells: s; for antibody and MMP-Inh.II inhibition p-values are shown.

Taken together, hypoxia supports an association between C4.4A and α6β4. Both molecules promote short term LN5 adhesion, but lasting migration. A serine protease and an MMP inhibitor restore adhesiveness. Migration is more strongly affected by an MMP inhibitor. Thus, hypoxia might be accompanied by increased protease activity accounting for LN5 degradation, where LN5 degradation products can exert chemotactic activity (Giannelli *et al.*, 1997;

Koshikawa *et al.,* 2000; Udayakumar *et al*., 2003). Alternatively, hypoxia might contribute to C4.4A shedding or release. These two possibilities are not mutually exclusive.

3.5 C4.4A cooperation with proteases

As a protease inhibitor interfered with ASML and Prog migration, I next tested whether under hypoxic condition LN5 becomes degraded and whether C4.4A is engaged in this process. ASML (Jung *et al.,* 2009) and Prog cells secrete LN5. C4.4A poorly co-localized with LN5 on BSA-coated plates under normoxic conditions. Co-localization was promoted, when $CoCl_2$-treated cells were seeded on LN5-coated plates (Fig. 14A). However, the experimental setting did not allow to judge on LN5 degradation. Therefore ASML and Prog cells were seeded in the presence or absence of $CoCl_2$ on uncoated plates, removing cells after 2d of culture by EDTA and staining for LN1, LN5 and FN. In $CoCl_2$-treated cultures the rim staining for LN5 was stronger and more focalized, but the area below the cell body was free of LN5. Instead, FN remained deposited below the cell body independent of $CoCl_2$-treatment. LN1 deposition was reduced, but not abolished. Staining of cell free areas confirmed poor LN5, high FN and intermediate LN1 recovery (Fig.14B).

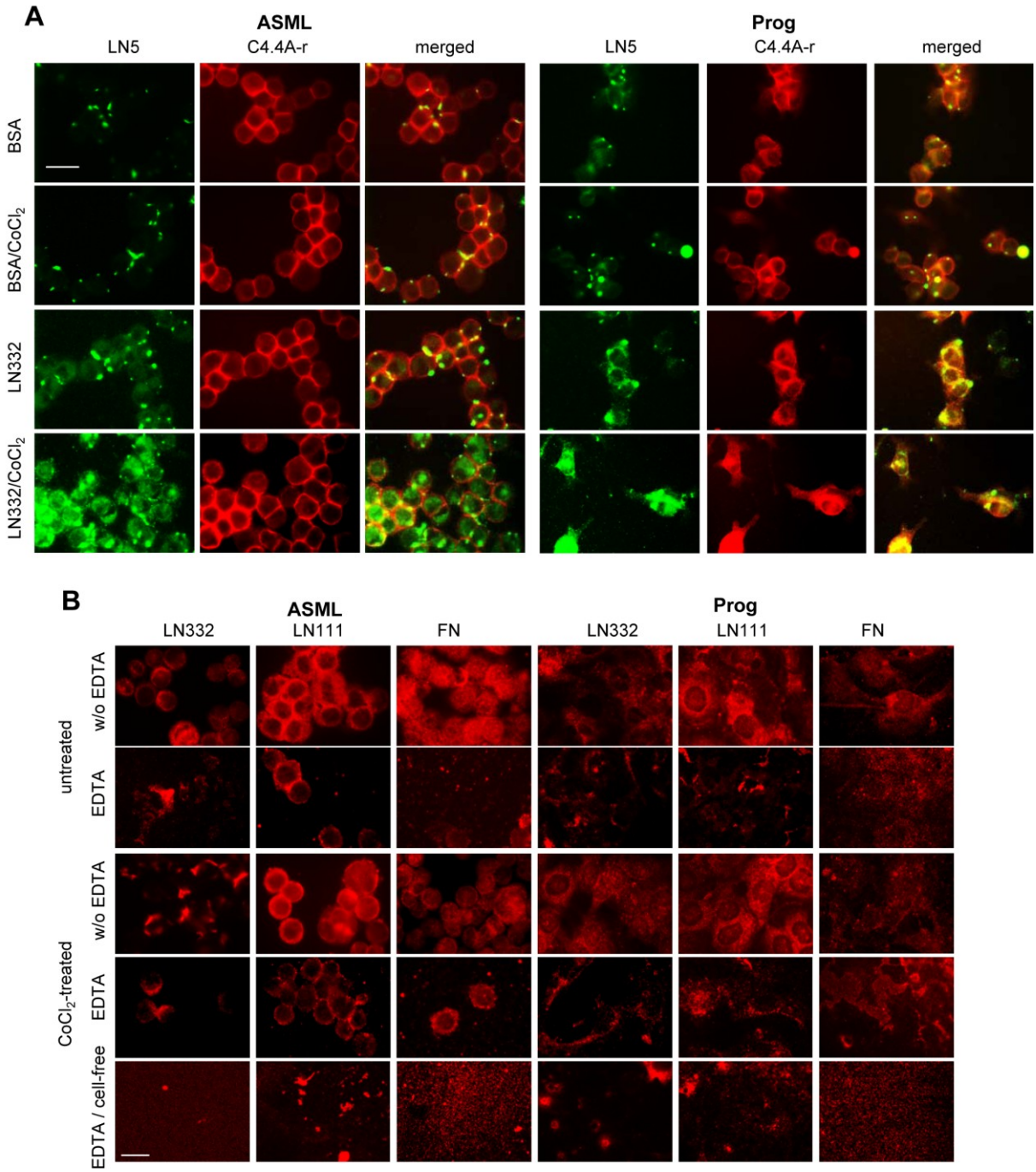


Figure 14: Cooperativity of C4.4A and MMP14 in LN5 degradation. (A) ASML and Prog cells cultured on LN5 (LN332)-coated cover slides in the absence or presence of $CoCl_2$ were fixed, stained with anti-LN5 / anti-mouse-Cy2 (g) and C4.4-TxR (r). Single fluorescence and digital overlays are shown. **(B)** ASML and Prog cells were cultured with or without $CoCl_2$ on glass slides. Cells, cell ghosts (EDTA) and areas where cells apparently were removed completely ("cell-free" area), were stained with anti-LNγ2, anti-LN1, anti-FN and Cy3-labeled secondary antibodies. Single fluorescence staining is shown (scale bar: 5µm).

These findings argued for LN5 and (partial) LN1 fragmentation. To control the hypothesis, LN1 and LN5 (804G supernatant) were co-cultured for 24h with

ASML cells in the presence of $CoCl_2$ with or without MMP-Inh.II. Cells were removed and the laminins were separated by SDS-PAGE. Gels were stained with Coomassie blue or proteins were transferred and blotted with anti-LN1 or anti-LNγ2 (anti-LN5). Coomassie blue staining provided evidence for LN1 and LN5 fragmentation, which was reduced, when cultures contained MMP-Inh.II. WB confirmed LNγ2 degradations and a weaker LN1 band was recovered, when LN1 was co-cultured with $CoCl_2$-treated ASML cells, but not when cultures contained MMP-Inh.II (Fig.15).

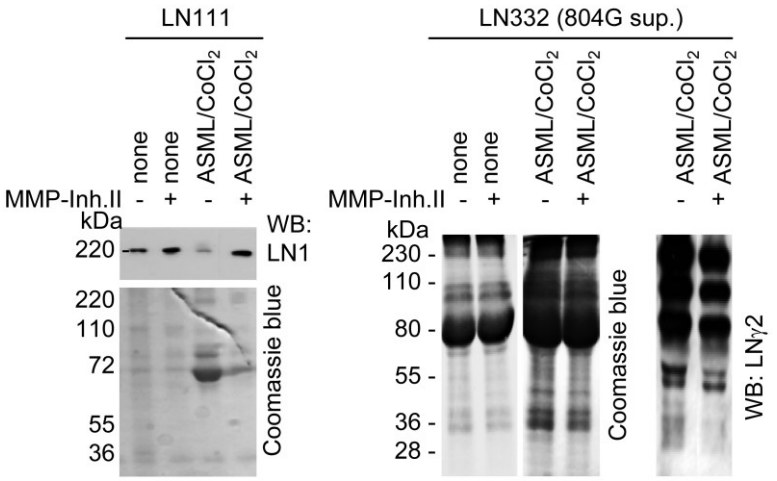


Figure 15: Cooperativity of C4.4A and MMP14 in LN1/LN5 degradation. ASML cells were suspended in 25µg LN1 (LN111) or 250µg 804G supernatant (LN332). Cells were incubated at 37°C for 16h in the presence of $CoCl_2$ with or without MMP-Inh.II. Cells were removed, supernatants were dried, dissolved in lysis buffer and separated by SDS-PAGE. Gels were stained with Coomassie blue or after transfer blotted with anti-ratLN1 or anti-ratLNγ2.

Our data showed that C4.4A might promote motility via associating with proteases involved in laminin degradation. According to the inhibitory activity of MMP-Inh.II, MMP14 was considered a possible candidate. Weak co-localization of MMP14 with C4.4A and α6β4 in resting cells became strong, when $CoCl_2$-treated cells were grown on LN5-coated slides (Fig.16A), which corresponded to an increase in co-immunoprecipitation of C4.4A and α6β4 with MMP14 in $CoCl_2$-treated ASML cells. C4.4A and more pronounced α6β4 also co-immunoprecipitated TACE, but not uPA, uPAR, MMP2 and MMP9. Although the C4.4A and α6β4 association with TACE was $CoCl_2$-treatment

independent, TACE clustering was more pronounced in $CoCl_2$-treated ASML cells (Fig.16B-16D).

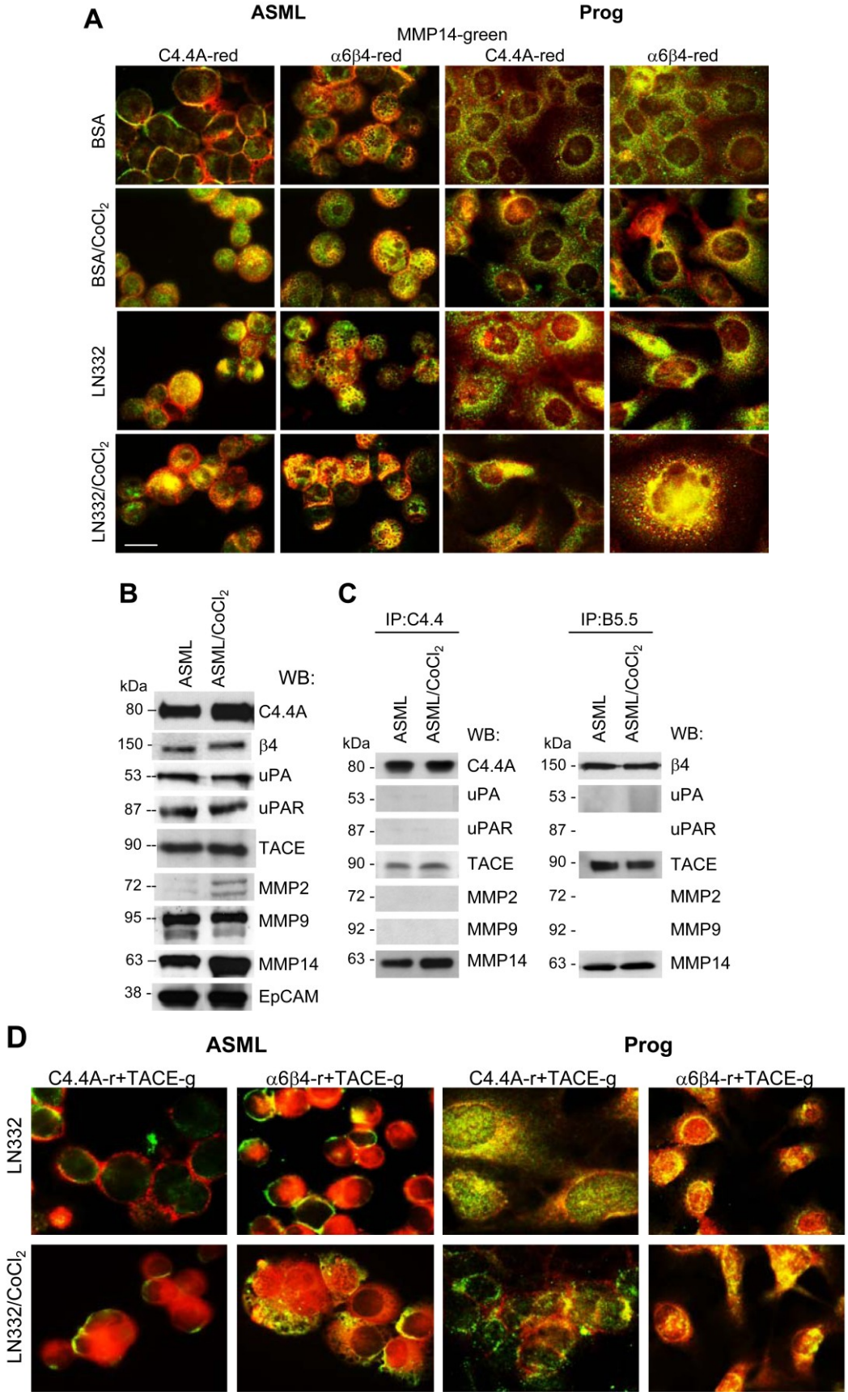


Figure 16: The C4.4A association with proteases. (A) ASML and Prog, grown on BSA- or LN5 (LN332)-coated plates in the presence or absence of $CoCl_2$ were stained with anti-MMP14 / anti-rabbit-Cy2 (g) and C4.4-TxR (r) or B5.5-TxR (r). Digital overlays are presented (bar size: 5µm). **(B)** Expression of C4.4A, α6β4, uPA, uPAR, TACE, MMP2, MMP9 and MMP14 in untreated and $CoCl_2$-treated ASML cells. **(C)** Co-immunoprecipitation of C4.4A and α6β4 with uPAR, TACE, MMP2, MMP9 and MMP14. **(D)** Co-localization of C4.4A and α6β4 with TACE in untreated and $CoCl_2$-treated ASML and Prog grown on LN5-coated glass slides. Digital overlays of staining with anti-TACE / anti-rabbit-Cy2 (g) and C4.4-TxR (r) or B5.5-TxR (r) are shown (bar size: 5µm).

The association of C4.4A with TACE, possibly via α6β4, and the fact that C4.4A has a highly sensitive cleavage site (Gårdsvoll *et al.,* 2007) and is delivered in exosomes (Paret *et al.,* 2005), prompted us to evaluate, whether $CoCl_2$-treatment supports C4.4A cleavage and/or exosomal release.

3.6 Functional activity of cell free C4.4A

C4.4A is a GPI-anchored molecule, which are located in rafts. Raft proteins are known to become internalized and to be recovered and enriched in exosomes, which contain functional active proteins. Thus, I next asked whether the C4.4A-MMP14-α6β4 complex is also present in exosomes. C4.4A is recovered in exosomes, a higher amount being recovered from $CoCl_2$-treated cells. Exosomes also contain α6β4. Exosomal C4.4A and α6β4 co-immunoprecipitate and also maintain the association with MMP14 and TACE, both MMP14 and TACE being known to be abundantly recovered in exosomes. I also recovered shed C4.4A, but only in the supernatant of $CoCl_2$-treated ASML cells. C4.4A shedding is largely abolished in the presence of TAPI. However, TAPI did not influence the delivery in exosomes. (Fig.17).

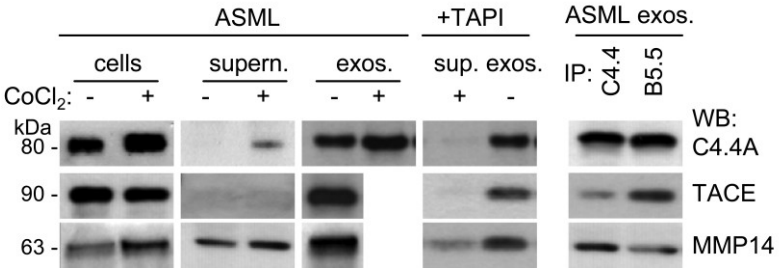


Figure 17: C4.4A recovery in exosomes and in vesicle-depleted culture supernatant. ASML cells were cultured in the absence of FCS with or without $CoCl_2$ and TAPI. Supernatant were collected and

separated by ultracentrifugation. Vesicle-depleted supernatant and lysed exosomes (20µg) were separated by SDS-PAGE, transferred and blotted with C4.4, anti-MMP14 and anti-TACE. In addition, precipitates of exosome lysates with C4.4 and B5.5 were separated by SDS-PAGE and after transfer blotted with C4.4, anti-MMP14 and anti-TACE.

These findings raised the question, whether exosomal or shed C4.4A would contribute to LN degradation. When exosomes and supernatant from $CoCl_2$-treated ASML cells were co-incubated with LN1 and LN5 (804G supernatant), exosomes, but not shed C4.4A degraded LN1 and LN5 as efficiently as ASML cells. Exosomes of $CoCl_2$-treated Colo357 cells were also incubated with LN5 (A431 supernatant). In comparison to the control (LN5 alone), LN5 is degraded by exosomes. LN degradation by exosomes was also inhibited by MMP-Inh.II (Fig.18).

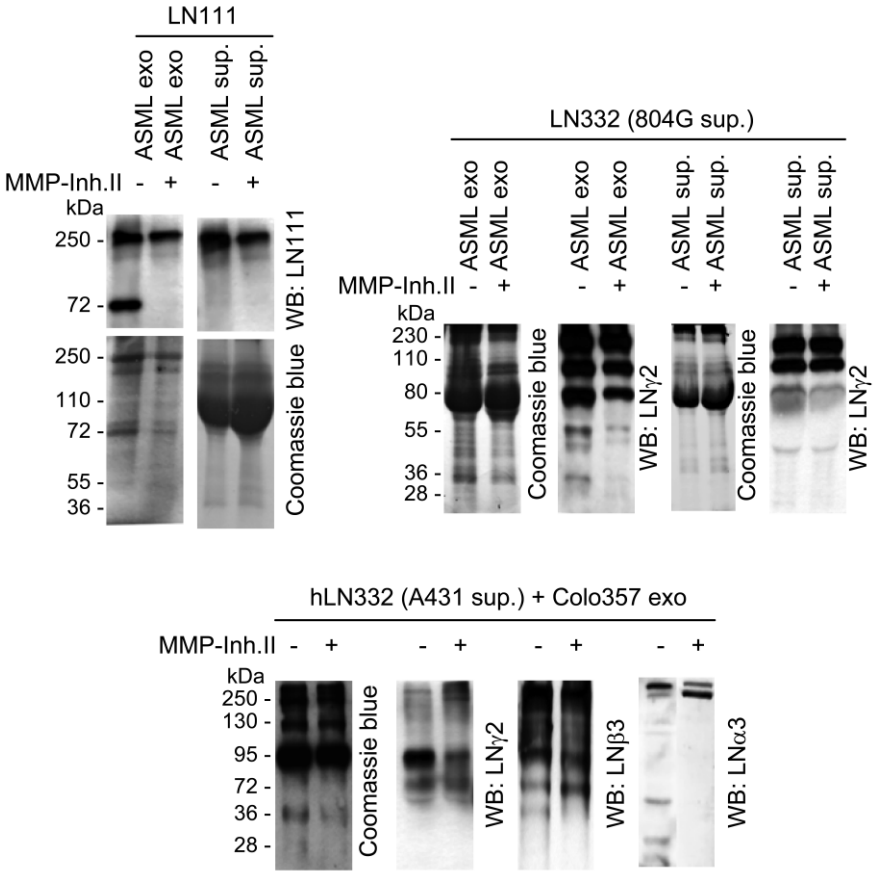


Figure 18: Laminin1 and laminin5 degradation by exosomes. Vesicle-depleted supernatant or exosomes of $CoCl_2$-treated ASML and Colo357 cells were incubated with 10µg/ml LN111 or 50µg 804G or A431 supernatant (LN332) in the presence or absence of MMP-Inh.II. After 16h incubation, cultures were dried and proteins dissolved in lysis buffer. Samples were separated by SDS-PAGE and gels were stained with Coomassie blue or proteins were transferred and stained with anti-ratLN1, -ratLNγ2, -humanLNα3, -humanLNβ3 or humanLNγ2.

Only supernatant of $CoCl_2$-treated ASML cells contained shed C4.4A, where TACE contributes to C4.4A shedding. Hypoxia strengthens incorporation of the functionally active C4.4A-α6β4-MMP14 complex into exosomes, such that LN1 and LN5 become degraded by exosomes.

3.7 C4.4A contribution to metastasis

C4.4A can associate with α6β4 and MMP14, and this complex contributes to matrix degradation and increased motility**.** To confirm the relevance of this association on metastasis formation *in vivo*, we generated a C4.4A^{kd} of the highly metastatic ASML line. To generate a C4.4A^{kd}, ASML cells were transfected with C4.4A siRNA and grown in Neomycin selection medium. Neomycin resistant cells were cloned by limiting dilution and tested for C4.4A expression by FACS after each recloning step until uniformly positive clones were obtained. For all the following experiments, we used clone1 (ASML-C4.4A^{kd1}) which showed a very strong reduction in C4.4A expression (Fig.19A). ASML and ASML-C4.4A^{kd} cells, were injected intrafootpad and tumor growth was followed until animals became moribund. Distinct to ASML^{wt} cells, ASML-C4.4A^{kd} cells transiently developed a small local tumor, but developed lymph node metastasis with a significant delay. After 44 days, when all ASML-bearing rats had become moribund, ASML-C4.4A^{kd}-bearing rats had not developed axillary or lung metastasis, which, however, were recovered with delay, when ASML-C4.4A^{kd} bearing rats became moribund. Due to the retarded metastatic spread, the mean survival time of ASML-C4.4A^{kd}-bearing rats was significantly prolonged from 39d of ASML-bearing rats to 64d (Fig.19B-19D).

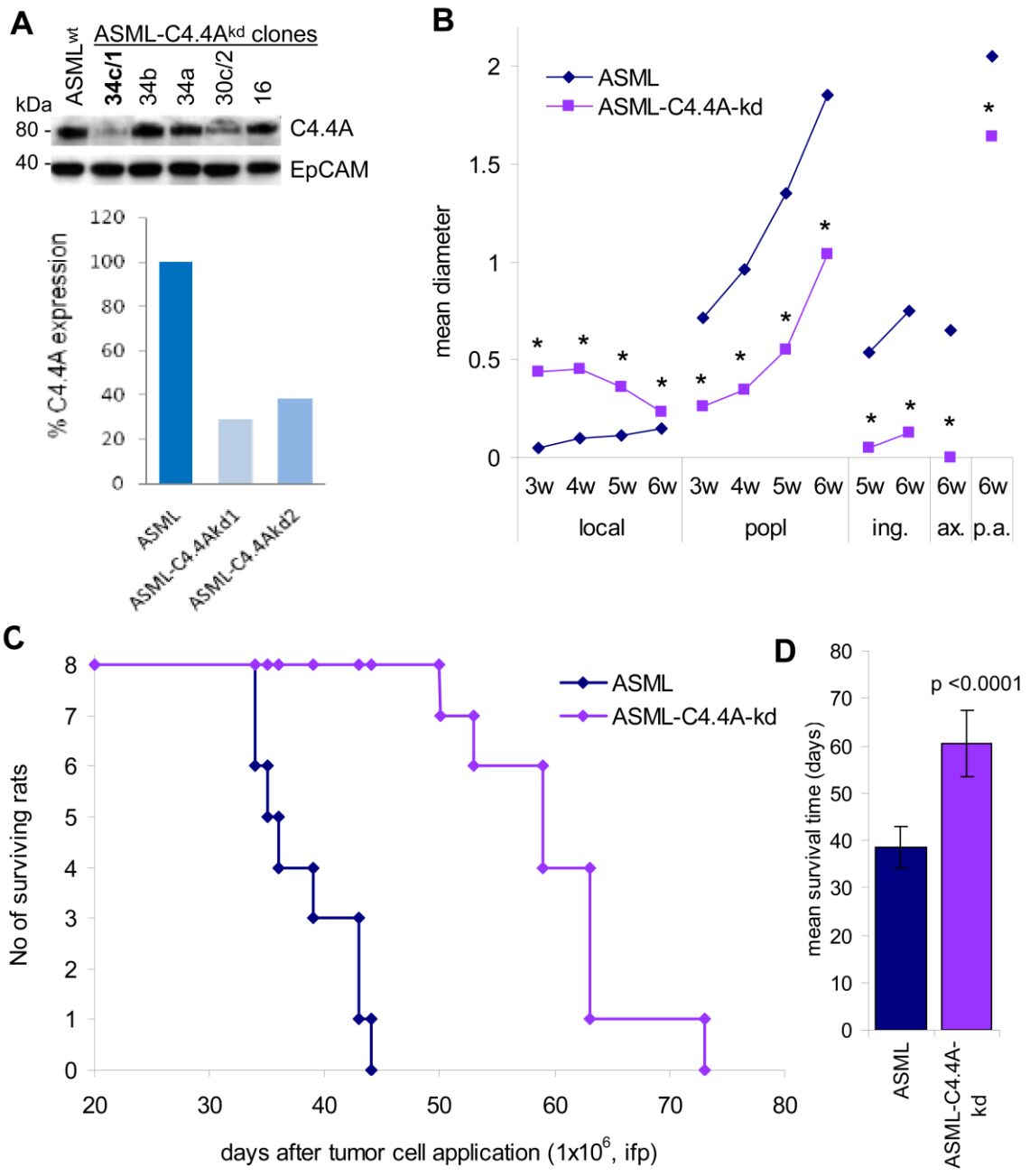
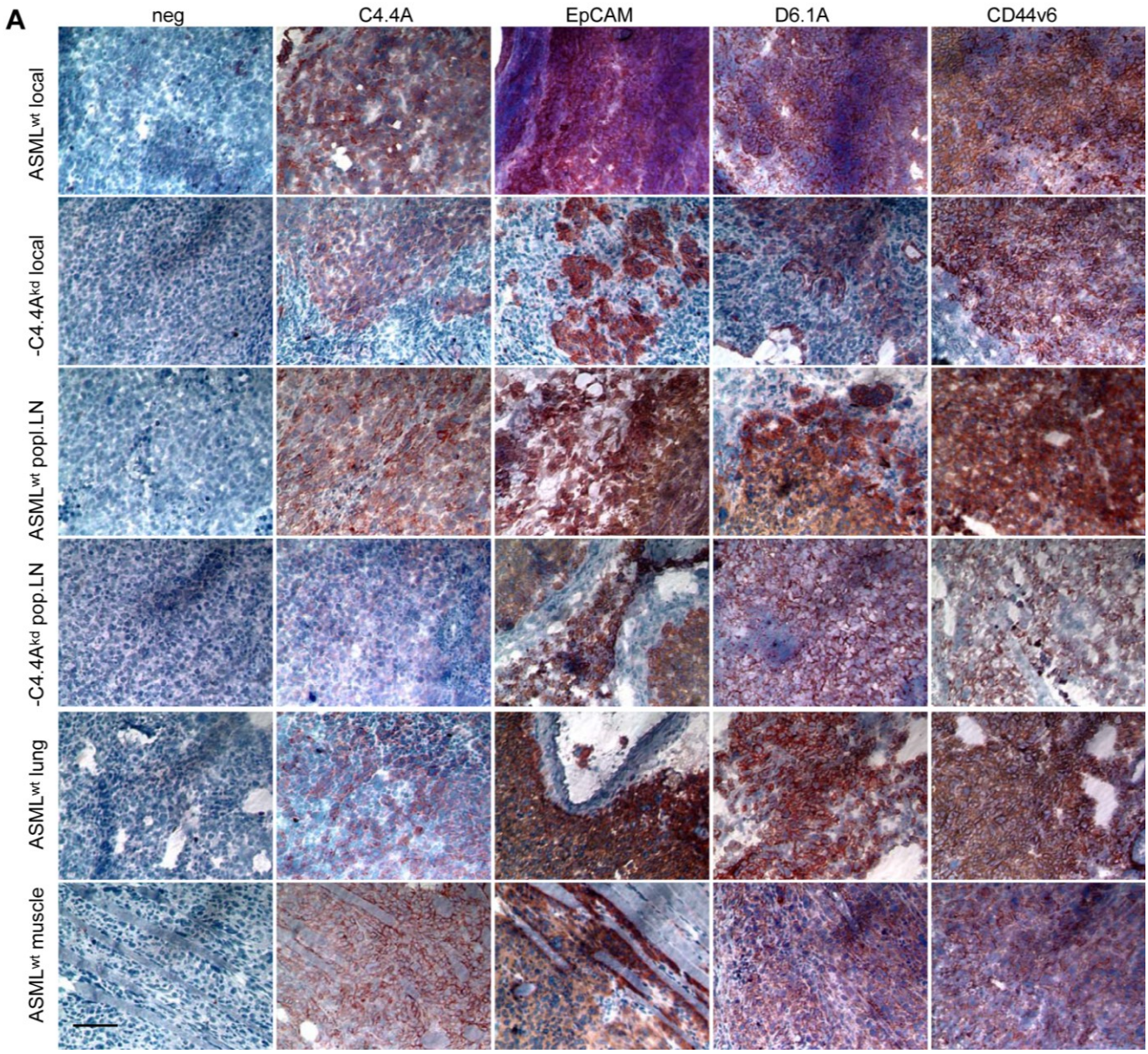


Figure 19: Retarded metastasis formation of ASML-C4.4A^{kd} cells. (A) WB of C4.4A in ASML and ASML-C4.4A^{kd} cells. EpCAM served as control. **(B-D)** BDX rats received 1x10^6 ASML or ASML-C4.4A^{kd} cells, ifp. **(B)** Local tumor growth and growth in draining (popliteal) and distant (inguinal, axillary, paraaortic <p.a.>) LN during 6wk after tumor cell application. The mean diameter of tumor of 5 rats / group is shown. **(C)** Survival time and survival rate of ASML and ASML-C4.4A^{kd} bearing rats **(D)** Mean survival time of 8 rats / group.

Immunohistology of local tumors, lymph node and lung metastasis, excised at late stages of tumor growth, confirmed a distinct growth profile of ASML and ASML-C4.4A^{kd} cells. While ASML cells grow dispersed between host cells, the ASML-C4.4A^{kd} cells form tumor clusters that do not penetrate the surrounding

tissue (Fig.20A-20B). In addition, ASML-C4.4A^{kd} tumors were rich in LN1 and LN5, particularly at the rim of the tumor nodules. Instead, LN1 and LN5 staining of ASML tumors was weaker and equally distributed (Fig.20C).



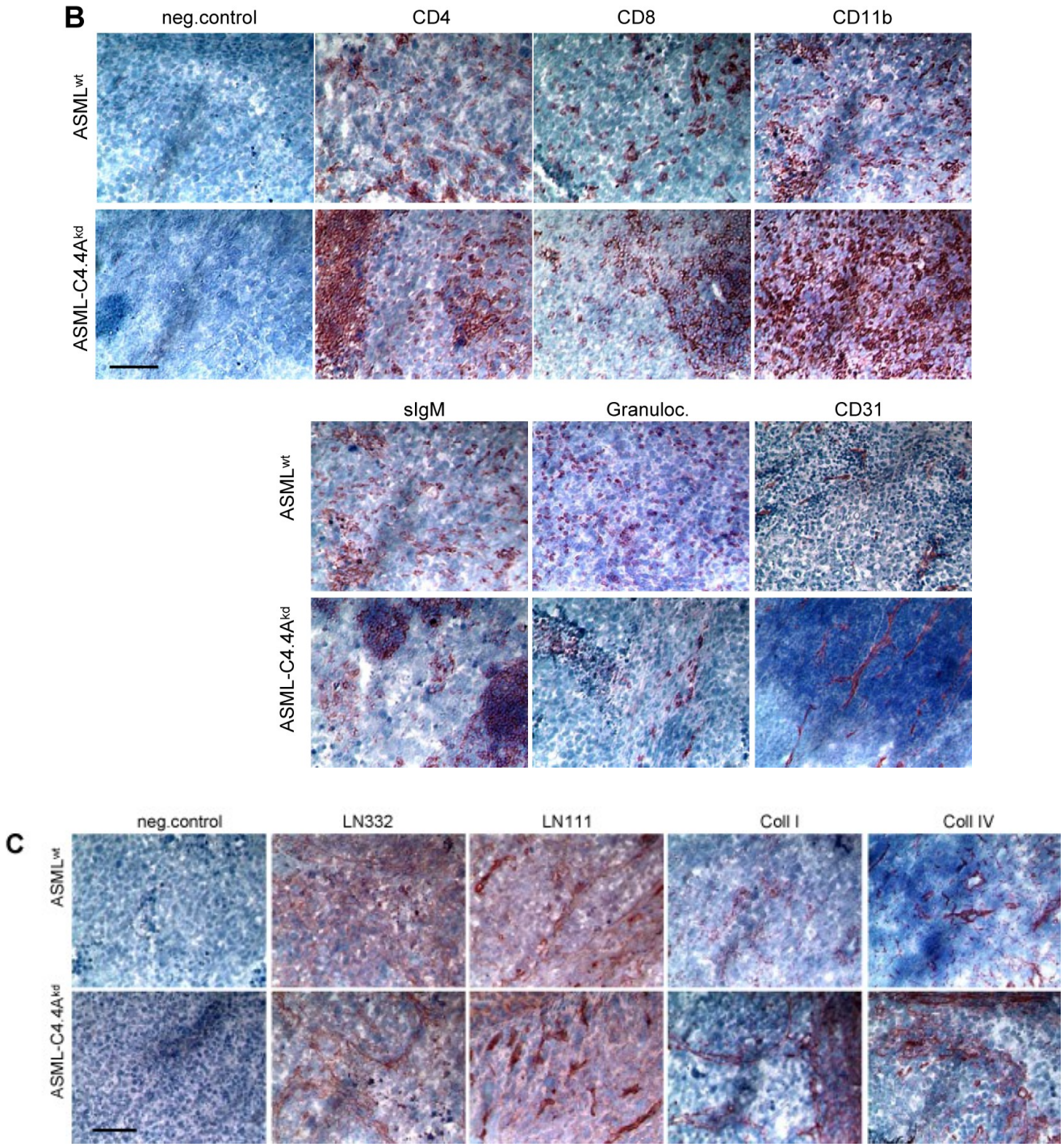
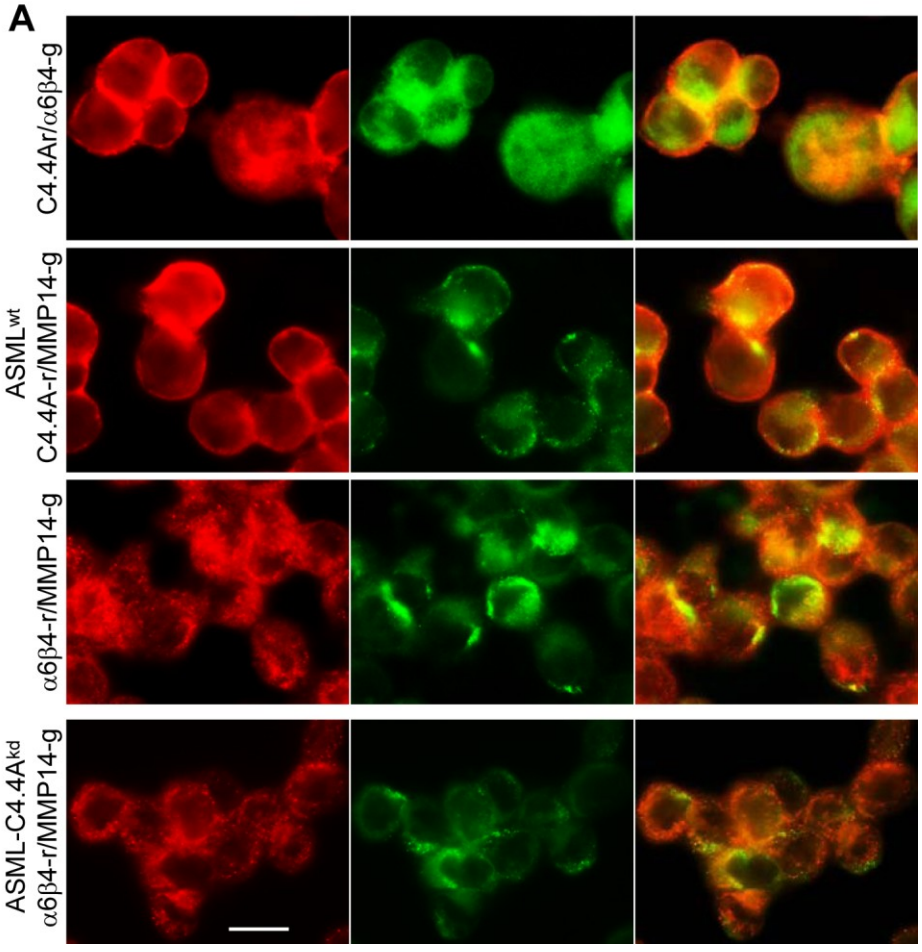


Figure 20: **Immunohistology of local and metastatic tissues after ASML or ASML-C4.4A^{kd} cells injection. (A)** Immunohistology of the local tumor, popliteal LN, lung and muscle metastasis of ASML and ASML-C4.4A^{kd}-bearing rats stained for the ASML markers C4.4A, EpCAM, D6.1A (Tspan8) and CD44v6 and **(B)** The popliteal node was stained with the leukocyte markers CD4, CD8 CD11b, sIgM and a granulocyte marker as well as with the endothelial marker CD31. **(C)** Immunohistology (popliteal LN) of the matrix proteins LN1 (LN111), LN5 (LN332), Coll I and Coll IV. (Scale bar: 100µm).

Thus, metastasis formation of ASML-C4.4A^{kd} cells is delayed and their capacity to invade surrounding tissue, including vessel endothelium, is strongly affected. This may be due to encapsulation by matrix proteins.

3.8 Motility / invasiveness reduction of ASML-C4.4A^{kd} cells is a sequel of impaired focalization of α6β4 and MMP14

We have shown before that upon stimulation C4.4A associates with α6β4 and MMP14, which promotes LN1 and LN5 degradation. Instead, when ASML-C4.4A^{kd} cells were seeded on LN1 or LN5 in the presence of PMA, α6β4 and MMP14 hardly co-localize (Fig 21A). Furthermore, only ASML, but not ASML-C4.4A^{kd} cells degraded LN1 and LN5, where degradation was inhibited in the presence of a broad range MMP inhibitor (Fig.21B).



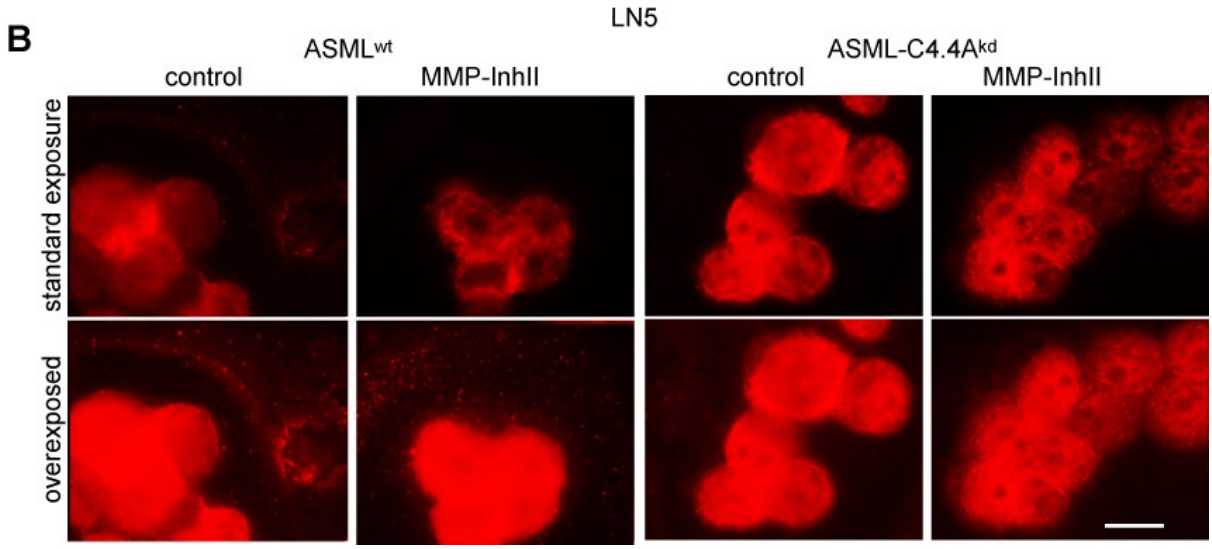


Figure 21: Cooperation of α6β4 and MMP14 in ASML-C4.4A^{kd} cells. (A) ASML and ASML-C4.4A^{kd} cells were seeded on LN5 (804G supernatant)-coated slides. Cells were double stained with C4.4A and α6β4 or MMP14 or with α6β4 and MMP14. Single fluorescence staining and digital overlays are shown (scale bar: 5µm). **(B)** ASML and ASML-C4.4A^{kd} cells were cultured overnight in the presence of PMA or DMSO (control) and MMP-InhII on glass cover slides. Cells were stained with anti-LNγ2. The standard exposure and overexposure are shown.

The failure of ASML-C4.4A^{kd} cells to recruit MMP14 towards α6β4 for LN degradation had severe consequences on cell migration. Transwell migration of ASML-C4.4A^{kd} cells was significantly reduced compared to ASML cell migration and was hardly inhibited by B5.5 (anti-α6β4). Furthermore, an MMP inhibitor had no effect on ASML-C4.4A^{kd} migration, whereas it strongly inhibited ASML^{wt} migration. A TACE inhibitor exerted no effect on both cell lines (Fig.22A, 22B).

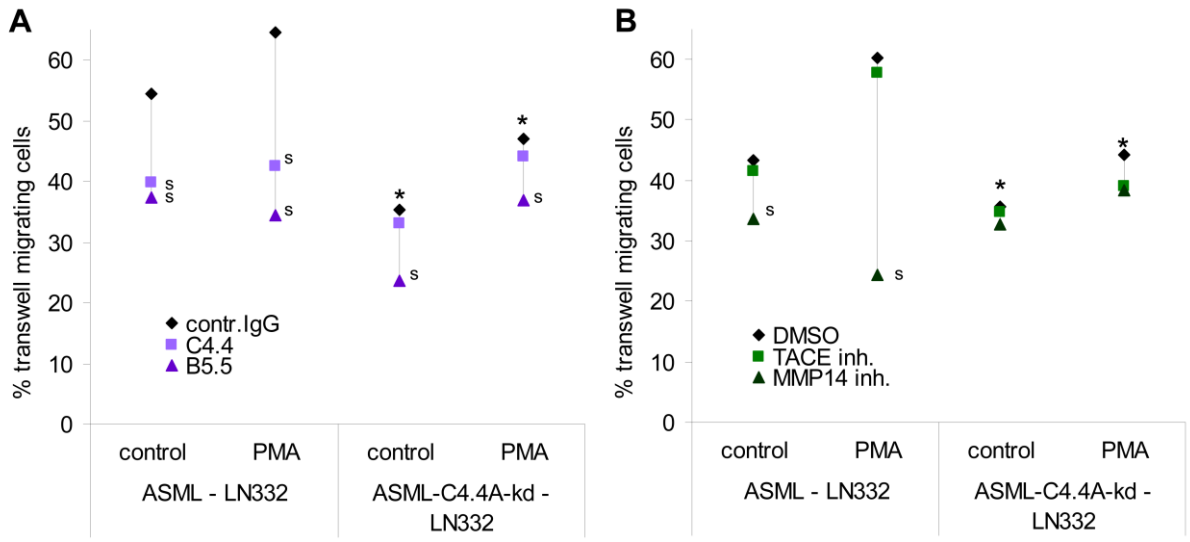
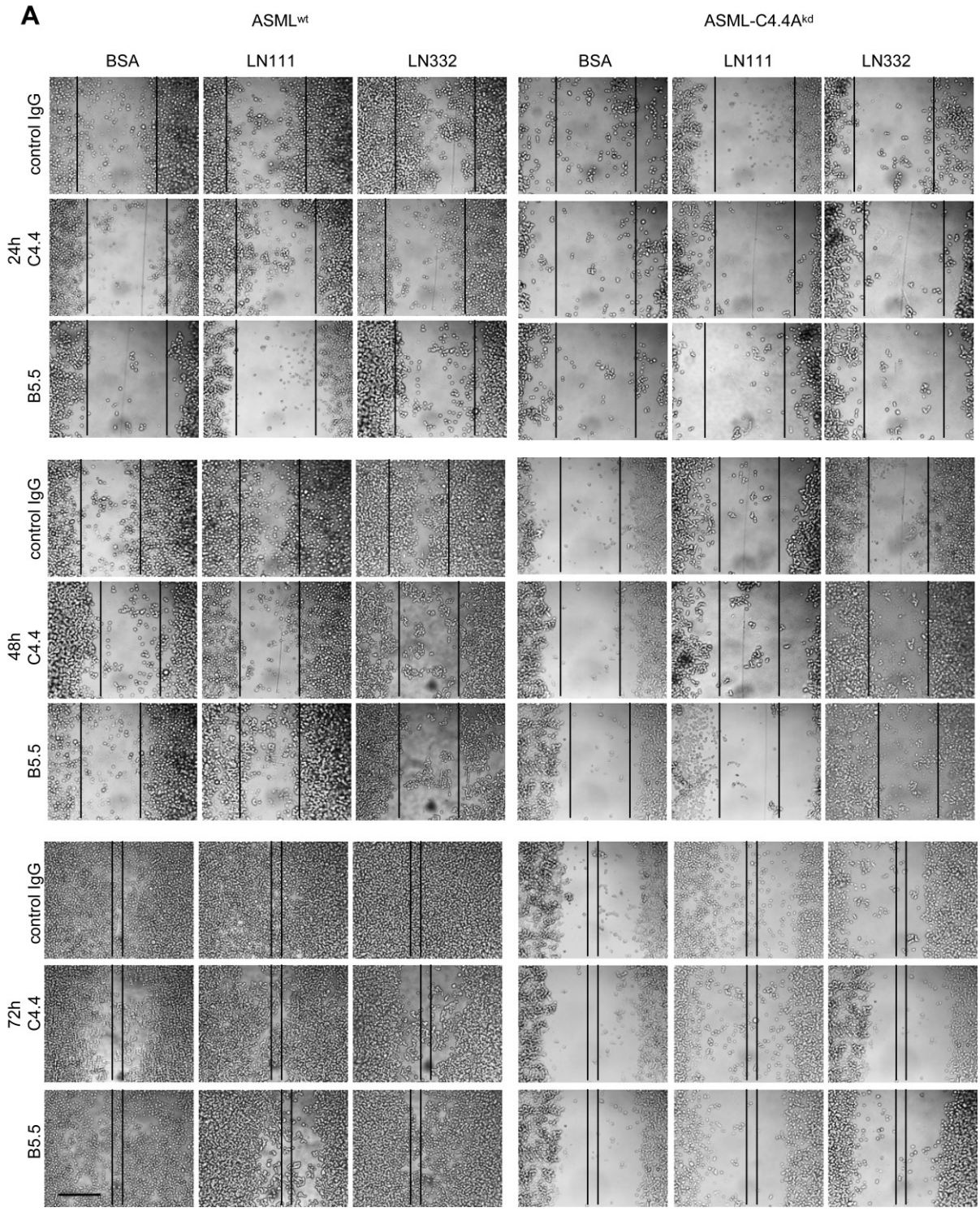


Figure 22: Impact of protease inhibitors on ASML-C4.4A^{kd} cell migration. (A) ASML and ASML-C4.4A^{kd} cells were untreated or PMA-treated and seeded in the upper part of a Boyden chamber. The lower chamber contained LN5 in RPMI/20%FCS. Where indicated cells were pre-incubated with C4.4 or B5.5. Migration was evaluated after 16h by staining the lower membrane site with crystal violet. The percent migrating cells (mean of triplicates) is shown. Significant differences between ASML and ASML-C4.4A^{kd} cells: *, significant antibody inhibition of migration: s. **(B)** ASML and ASML-C4.4A^{kd} cells were incubated with TAPI (TACE inhibitor) or MMP-InhII (MMP14 inhibitor) and seeded in the upper part of a Boyden chamber. Migration was evaluated as above. Significant differences between ASML and ASML-C4.4A^{kd} cells: *, significant differences in the presence of the inhibitors: s.

The cooperativity between C4.4A and α6β4 was also confirmed in an *in vitro* wound healing assay, where C4.4 and B5.5 inhibited ASML cell migration, but neither antibody inhibited the poor migration of ASML-C4.4A^{kd} cells (Fig.23).


A
ASML^wt
ASML-C4.4A^kd
BSA
LN111
LN332
BSA
LN111
LN332
control IgG
24h C4.4
B5.5
control IgG
48h C4.4
B5.5
control IgG
72h C4.4
B5.5

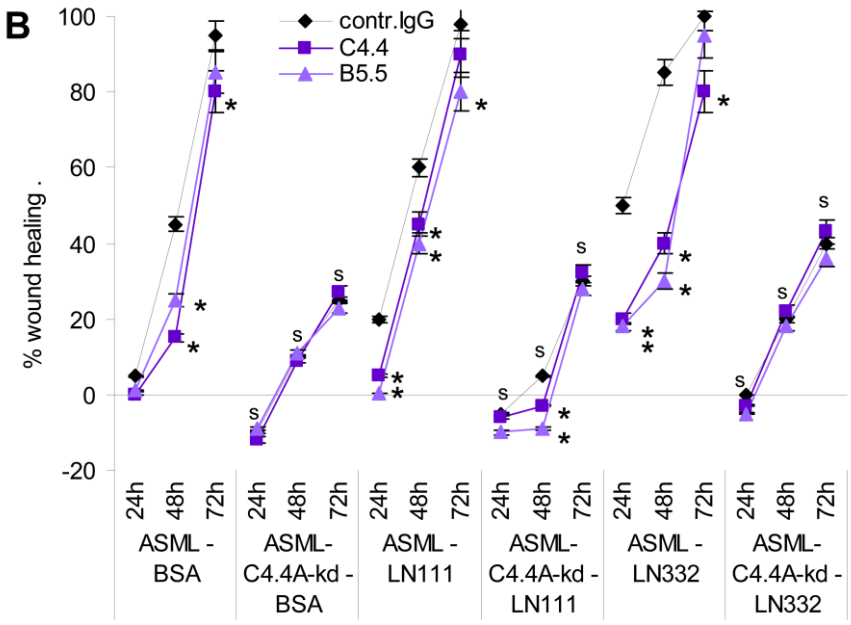


Figure 23: Impact of anti-α6β4 on wound healing of ASML-C4.4A^{kd} cells. ASML and ASML-C4.4A^{kd} cells were seeded in 24-well plates coated with BSA, LN111 or LN223. **(A, B)** When reaching subconfluence, the monolayer was scratched. Wound healing was evaluated for 72h by light microscopy. Where indicated, the cultures contained C4.4 or B5.5. **(A)** A representative example and **(B)** the mean % of wound closure are shown. Significant differences between ASML and ASML-C4.4A^{kd} cells: *; significant differences between control IgG, C4.4 and B5.5: s. (Scale bar: 50µm)

Taken together, co-localization of α6β4 and MMP14 is reduced in PMA-stimulated ASML-C4.4A^{kd} cells. This is accompanied by a reduction in LN5 degradation and in migratory activity.

3.9 Impaired drug resistance of ASML-C4.4A^{kd} cells

To control whether the retarded tumor growth is due only to impaired motility, a second *in vivo* experiment was performed, where tumor cells were injected intraperitoneally such that at least intraperitoneal tumor growth should not be affected by impaired motility. While the mean survival time of rats receiving 5x10^6 ASML^{wt} cells was 20.4±0.5 days, the survival time of ASML-C4.4A^{kd}-bearing rats differed with 37±9.7 days significantly. Furthermore, while the survival time of ASML^{wt}-bearing rats was not significantly prolonged by

cisplatin-treatment, cisplatin-treated ASML-C4.4A^{kd}-bearing rats are still alive (Fig.24).

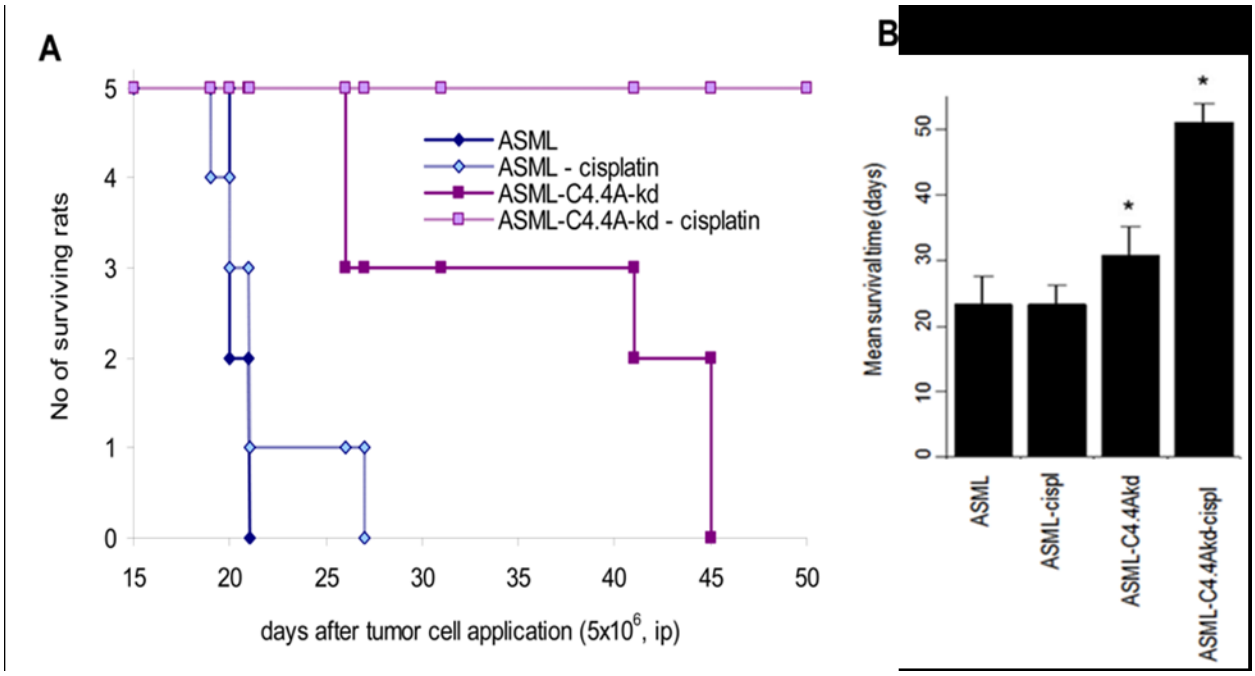


Figure 24: Cisplatin susceptibility of ASML-C4.4A^{kd} cells: BDX rats received 5x10^6 ASML or ASML-C4.4A^{kd} cells, ip. After 2 and 23 days, they received 1µg/kg cisplatin, ip. (A) Survival time and rate of 5 rats / group; (B) Mean survival time. Significant differences between ASML and ASML-C4.4A^{kd}- bearing rats or ASML-cisplatin and ASML-C4.4A^{kd} -cisplatin-bearing rats : *

One possible reason could have been a loss in apoptosis resistance, where it should be noted that ASML^{wt} are known to be extremely apoptosis resistant (Matzku et al.,1983; Klingbeil et al., 2009). To control for the hypothesis, I evaluated apoptosis resistance *in vitro* in the presence of cisplatin. In fact, 7.5µg/ml cisplatin sufficed for a 50% reduction of the proliferative activity of ASML-C4.4A^{kd} cells as compared to ASML^{wt} cells, which required 20µg/ml cisplatin. Similar effects were seen evaluating the percentage of apoptotic cells by AnnexinV/PI staining. The difference in cisplatin resistance between ASML-C4.4A^{kd} and ASML^{wt} cells was even more striking, evaluating anchorage-independent growth. Only 0.6% of ASML-C4.4A^{kd} cells, but 30% of ASML^{wt} cells formed colonies in the presence of 1µg/ml cisplatin (Fig.25A-25D).

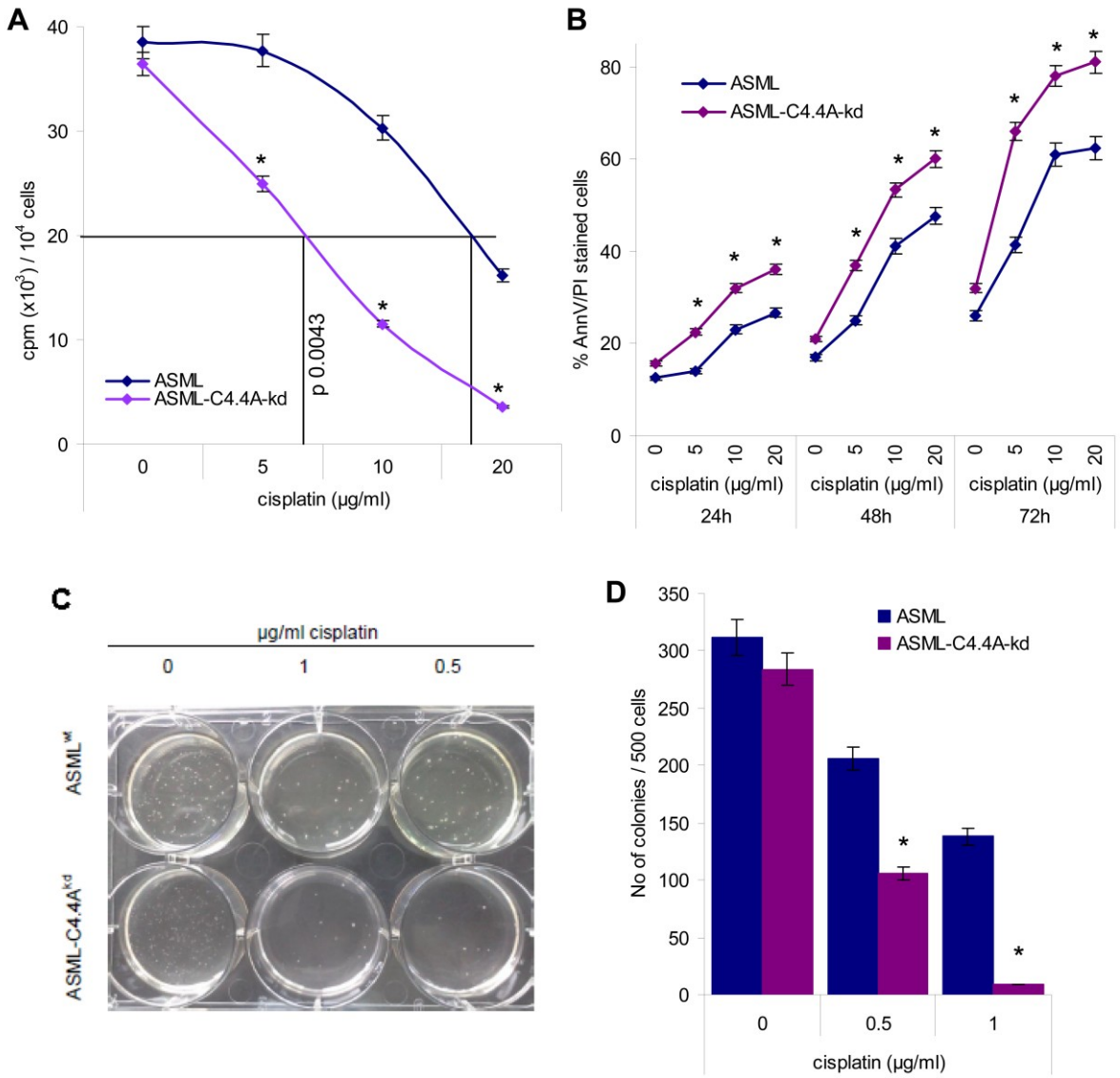


Figure 25: Reduced apoptosis resistance of ASML-C4.4A^{kd} cells. (A-D) ASML and ASML-C4.4A^{kd} cells were cultured with titrated amounts of cisplatin. **(A)** ^{3}H-thymidine incorporation, **(B)** AnnexinV/PI staining, **(C, D)** soft agar colony formation. **(C)** Representative example. **(D)** Mean ± SD of colonies in soft agar. Significant differences in cisplatin resistance between ASML and ASML-C4.4A^{kd} cells: *

ASML-C4.4A^{kd} cells show a significant decrease in apoptosis resistance, most strongly in anchorage-independent growth.

To evaluate the underlying mechanism, I started to control for caspase activation, where caspase 8 is triggered by death receptors. Caspase 9 and caspase 3 become activated downstream of caspase 8 or via the mitochondrial apoptosis pathway. A flow cytometry analysis of cisplatin-treated ASML^{wt} and ASML-C4.4A^{kd} cells provided clear evidence for unaltered caspase 8 activity,

but a strong upregulation of cleaved caspase 9 and activated caspase 3 in cisplatin-treated ASML-C4.4A^{kd} cells (Fig. 26).

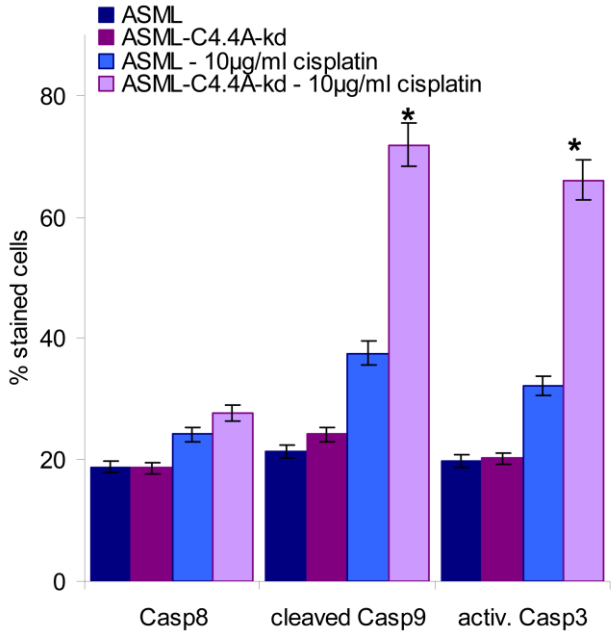


Figure 26: Caspase activity in ASML^{wt} and ASML-C4.4A^{kd} cells. ASML and ASML-C4.4A^{kd} cells were cultured in the presence or absence of cisplatin (10µg/ml). Cells were stained with anti-caspase 8, -cleaved caspase9 and -activated caspase 3 and the appropriate secondary antibody. Expression was evaluated by FACS. Significant differences in caspase activity between cisplatin-treated ASML and -ASML-C4.4A^{kd} cells: *

This finding excludes receptor-mediated apoptosis accounting for the pronounced cisplatin sensitivity of ASML^{wt} cells. The definition of changes in the mitochondrial pathway of apoptosis remains to be specified.

In brief, C4.4A expression is regulated by stress, including hypoxia. It contributes to cell motility by associating with α6β4 in rafts and by forming a complex with MMP14. Thereby, MMP14 becomes triggered to degrade LN5, the major ligand of C4.4A. These findings were confirmed in a C4.4A^{kd} line. The ASML-C4.4A^{kd} line provided additional evidence for a contribution of C4.4A to apoptosis resistance. The deficiency of C4.4A does not affect death receptor-mediated apoptosis, but rather the mitochondrial pathways of apoptosis resistance. Loss of apoptosis resistance in ASML-C4.4A^{kd} cells may also well rely on the failure to recruit α6β4 towards raft located C4.4A.

4. DISCUSSION

The metastatic cascade demands particular features from a tumor cell. Besides of the capacity to isolate from the primary tumor mass, migratory, as well as adhesion features are required. Tumor cells should have invasive potential and be apoptosis resistant to survive in the isolated state and in the foreign environment (Mina and Sledge, 2011).

C4.4A is a metastasis-associated protein, which was first identified in a screen designed to select membrane proteins that were differentially expressed by highly metastasizing rat pancreatic cell lines (Claas *et al*., 1996; Matzku *et al*., 1989). The function of metastasis-associated C4.4A, with very restricted expression in normal tissue (Rösel *et al*., 1998; Würfel *et al*., 2001; Hansen *et al*., 2004; Claas *et al*., 1996; Smith *et al*., 2001), remains elusive (Jacobsen *et al*., 2008). Nonetheless, circumstantial evidence has prompted speculations that C4.4A, which is a receptor for LN1 and LN5, may be involved in either cell-matrix interactions (Rösel *et al*., 1998; Smith *et al*., 2001; Paret *et al*., 2005) or in cell-cell adhesion (Hansen *et al.*, 2008).

Invasion and metastasis are the major characteristics of malignant tumors (Ryu *et al*., 2010), where one of the common features is a state where oxygen tension drops below normal limits (Vaupel *et al*., 2004). Here, we report that hypoxia-induced C4.4A up-regulation promotes transition from a sessile towards a mobile phenotype through pronounced association with the integrin α6β4 and proteases, where the latter strengthens motility by focalized matrix degradation. Motility and invasiveness are further strengthened by the release of a C4.4A-α6β4-MMP14 complex into exosomes, which extends the range of matrix degradation as exosomes are known to contain functionally active proteins (André *et al*., 2002; Zöller, 2006; Schorey and Bhatnagar, 2008).

After having explored the functional consequences of C4.4A up-regulation under hypoxia, I controlled these findings in a knock down C4.4A in a metastasizing tumor cell line created by the siRNA approach. *In vitro* and *in vivo* studies confirmed the involvement of C4.4A in cell motility and invasiveness. In addition, the C4.4A knockdown provided evidence that C4.4A also contributes to apoptosis/cytotoxic drug resistance.

Thus, there is strong evidence that C4.4A acts as a metastasis supporting molecule. However, none of the described features are genuine characteristics of C4.4A. Instead, C4.4A co-ordinates the association with α6β4 and MMP14. α6β4 promotes motility and apoptosis resistance. MMP14 facilitates matrix degradation and invasiveness. I will discuss, how C4.4A binding to LN1 and LN5 acts as a linker that co-ordinates several steps in the metastatic cascade.

4.1 Regulation of C4.4A expression in hypoxia

Low oxygen level and, as a surrogate, $CoCl_2$-treatment (Liu *et al*., 1999), are accompanied by HIF1α accumulation, that becomes transported to the nucleus, where it acts as a transcription factor for multiple genes (Yee *et al*., 2008; Brahimi-Horn and Pouysségur, 2009). Several studies have shown that hypoxia causes HIF1α accumulation in several human malignancies. I first demonstrated that hypoxia is accompanied by up-regulated C4.4A expression. As the *C4.4A* promoter contains 3 HRE, we considered the possibility that HIF1α may contribute to *C4.4A* transcription by binding to the HRE to drive its transcription. However, co-transfection with HIF1α expression vector as well as transfection with the C4.4A promoter bearing the HRE mutation, did not provide evidence for HIF1α contributing to *C4.4A* transcription. C/EBPβ, essentially required for C4.4A transcription (Fries *et al*., 2007), has also been associated with metastasis-associated gene transcription (Chun *et al*., 2004), gene expression in differentiating keratinocytes and during wound repair

(Maytin *et al*., 1999; House *et al*., 2010). However, we did not observe significantly up-regulated *C4.4A* transcription in $CoCl_2$-treated cells. Thus, hypoxia-induced high C4.4A expression may be a consequence of stabilized protein expression or increased expression of its associated partners. The finding that $CoCl_2$-treatment does not induce expression in C4.4A-negative tumor lines is in line with this interpretation. Nonetheless, hypoxia is accompanied by an increase in C4.4A expression, which allowed me to proceed in evaluating its functional activities.

4.2 Cooperation of C4.4A with α6β4 and MMP14

C4.4A expression becomes strongly up-regulated in migrating keratinocytes during wound repair (Paret *et al.,* 2007) and a direct contribution to keratinocyte migration has been demonstrated by anti-C4.4A antibody significantly inhibiting wound healing. *In vitro*, $CoCl_2$-treatment promoted migration of $C4.4A^+$ tumor cells on LN5, a major C4.4A ligand (Paret *et al*., 2005), after a transient increase in LN5 adhesion.

The engagement of C4.4A in the shift towards motility on LN5 relies on hypoxia-induced associations of C4.4A with α6β4 and MMP14. On LN5, but not on BSA, C4.4A co-localizes and associates with α6β4, focalized co-localization and association being stronger under hypoxia. LN5 is a major ligand for α6β4 (Rabinovitz , Mercurio *et al*., 1996; Kariya *et al*., 2009) and one could have speculated that C4.4A only acts as an accessory molecule. This has been excluded as recombinant C4.4A binds LN5 (Paret *et al*., 2005) and AS1B1 cells, which do not express α6β4 (Rösel *et al.*, 1998), also show pronounced LN5 binding. Finally, adhesion and migration on LN5 became equally well inhibited by C4.4 and anti-α6β4. Whether C4.4A recruits α6β4 or vice versa or whether α6β4 and C4.4A come into vicinity via their joint ligand(s), remains to be explored.

Though LN5 can account for stable anchoring contacts through interaction with α6β4 and the β4 chain-initiated recruitment of collagen XVII and plectin (Litjens *et al*., 2006), LN5 also stimulates directed cell migration (Guess and Quaranta, 2009) mostly through rac1 activation (Pullar *et al*., 2006). Thus, the C4.4A-α6β4 association and joint binding to LN5 can well account for increased migration during hypoxia. Migration-promoting activity of LN5 is also strengthened by proteolytic processing (Marinkovich *et al*., 1992), where the rat LNγ2 chain is cleaved by MMP2 and MMP14 (Koshikawa *et al*., 2000; Giannelli *et al*., 1997). One of the γ2 fragments activates the EGFR (Schenk *et al*., 2003). Different LN5 fragments from the γ2 and the α3 chain also account for the deposition of LN into the matrix underlying cultured cells (Gagnoux-Palacios *et al.*, 2001; Sigle *et al*., 2004). MMP14 and MMP7 can also cleave the β3 chain (Udayakumar *et al.,* 2003; Remy *et al*., 2006); the α3 chain can be cleaved by several proteases including plasmin (Ogura *et al*., 2008). Three observations are in line with hypoxia-induced C4.4A up-regulation accounting for pronounced migration due to LN5 fragmentation: (i) broad serine or MMP inhibitors promoted LN5 adhesion; (ii) LN5 deposits were not recovered in $CoCl_2$ treated cultures; (iii) an MMP inhibitor strongly reduced migration on LN5. As aprotinin partially restored adhesion to LN5, but MMP-Inh.II exerted stronger effects on cell migration, we tested several proteases for associating with C4.4A. C4.4A does not associate with uPAR, uPA, MMP2 or MMP9, but associates with TACE/ADAM17 and, more strongly in hypoxia with MMP14. The fact that C4.4A can be cleaved by TACE (Esselens *et al*., 2008) and LN5 can be degraded by MMP14 (Tsuruta *et al*., 2008) is in conformity with the association of C4.4A with TACE and MMP14.

The association of C4.4A with α6β4 and MMP14, which allows for focalized LN5 fragmentation that can promote directed motility, provides a first explanation for the activity of C4.4A in tissue remodeling, wound repair and tumor progression.

4.3 Hypoxia and C4.4A release

C4.4A shedding and exosomal release (Paret *et al*., 2007) are both stimulated under stress, including hypoxia. Soluble C4.4A has only been detected under hypoxia and could be linked to C4.4A cleavage by TACE/ADAM17, as already reported (Esselens *et al*., 2008). The increase in exosomal C4.4A corresponds to a pronounced exosome release under stress (Simons *et al*., 2009). In line with the internalization of membrane microdomains, such that internalized protein complexes remain intact (Staubach *et al*., 2009), exosomes collected from $CoCl_2$-treated tumor cells contained C4.4A, α6β4 and MMP14, where for the latter clathrin- and caveolin-dependent internalization has been described (Jiang *et al*., 2001). Exosomal proteins are functionally active (Escrevente *et al*., 2008), which also accounts for MMP14 (Hakulinen *et al*., 2008). In fact, the exosomal C4.4A-α6β4-MMP14 complex, but not soluble C4.4A suffices for LN5 degradation. These findings uncovered two important points. First, the activity of the C4.4A-α6β4-MMP14 complex is not restricted to the cell membrane, but can additionally prepare the surrounding of migrating epithelial and tumor cells, thus extending the operational range of C4.4A. Second, the finding that shed C4.4A does not degrade LN5 confirms that not C4.4A by itself, but a complex of C4.4A with proteases is required for LN5 fragmentation.

Taken together, C4.4A up-regulation induced by hypoxia is accompanied by transiently increased LN5-adhesion and pronounced migration. LN5 adhesion and migration are supported by the strong C4.4A-α6β4 association under hypoxia. The shift towards migration becomes facilitated by focalized LN5 degradation by membrane-bound and exosomal C4.4A-associated MMP14. The recovery of a functionally active C4.4A-α6β4-MMP14 complex in exosomes adds to the functional relevance of C4.4A in wound healing, tissue remodeling and tumor cell spread.

4.4 The impact of C4.4A on metastasis formation

C4.4A has originally been detected on a metastasizing rat adenocarcinoma (Matzku *et al*., 1989; Claas *et al*., 1996) and thereafter has been detected in several human cancer, where C4.4A expression frequently was more pronounced or even restricted to metastatic tissue (reviewed in Jacobsen and Ploug, 2008). However, when a non-metastasizing subline of the rat pancreatic adenocarcinoma, the AS line was transfected with C4.4A, it did not metastasize after subcutaneous application, whereas after iv injection AS as well as AS-1B1 cells grow in the lung. Distinct to AS cells, AS-1B1 tumors do not form a fibrous capsule and grow aggressively with destruction of bronchioli and vessel walls. The findings with the ASML-C4.4A^{kd} are in line with these features. ASML-C4.4A^{kd} cells are still capable to metastasize via the lymphatic system, albeit with a significant delay. However, the growth profile of ASML-C4.4A^{kd} tumors differs from that of ASML^{wt} tumors in several aspects: (i) the tumor grows locally before metastases develop; (ii) the local tumor and metastases have a fibrous capsule; (iii) in line with this, no dispersed tumor cells are detected in the surrounding tissue and (iv) distinct from ASML^{wt} cells, ASML-C4.4A^{kd} cells show well developed tumor vessels.

The local growth, the fibrous capsule, the failure to invade the surrounding tissue and the recovery of intact vessels in tumors could well rely on the association of C4.4A with MMP14 and, only under stress conditions, the recruitment of α6β4 towards the raft located C4.4A.

I already described that stress-induced activation of C4.4A supports the proteolytic activity of MMP14. Thus low C4.4A expression in ASML-C4.4A^{kd} cells obviously does not suffice for MMP14 activation and hence, LN degradation is decreased. MMP14 also account for MMP2 activation (Holmbeck *et al*, 2004), which predominantly degrades collagens (Schneider *et al*, 2008). Impaired MMP2 activation can further account for encapsulation of the ASML-

C4.4A^{kd} tumor. Undisturbed encapsulation of ASML-C4.4A^{kd} tumors, in turn, explains that the tumor cells do not penetrate into the surrounding tissue. Though the better vascularization of ASML-C4.4A^{kd} tumors could be a response to the demand of the solid tumor mass for oxygen, we consider it more likely that the poor vascularization of ASML^{wt} tumors is a consequence of overshooting matrix degradation, thus destroying the path for sprouting capillary. This will not take place in ASML-C4.4A^{kd} tumors. The presence of short capillary fragments in the ASML^{wt} tumor supports our interpretation.

Taken together, although we did not recover any direct functional activity of C4.4A besides binding to LN5, LN1 and galectin3 (Paret et al., 2005), by its raft localization and the activation induced recruitment of α6β4 as well as the associated activation of raft-located MMP14, C4.4A becomes central in coordinating tumor cell motility and invasiveness. The finding that in esophageal cancer C4.4A expression is reduced at early stages of tumor growth and becomes upregulated during metasasis formation (Hansen *et al*, 2008), supports the concept of a tightly regulated expression that selectively supports migration and invasion of isolated or leading front tumor cells by associating with activated α6β4 and MMP14, which allows for cytoskeleton reorganization and for creating space, where LN5 track formation (Sehgal *et al.*, 2006) as well as short fragments of LNγ3 (Decline and Rousselle, 2001) can strengthen motility.

4.5 The contribution of C4.4A to drug resistance

Compared to ASML^{wt} cells, ASML-C4.4A^{kd} tumor cells are highly susceptible to cisplatin, which *in vivo* allows for a significant prolongation of the survival time. We used cisplatin as a therapeutic at a relatively low dose that does not exert cytotoxic side effects. In the absence of cisplatin, there has been no evidence for reduced apoptosis resistance of ASML-C4.4A^{kd} compared to

ASML^{wt} cells. This again pointed towards a stress situation, where C4.4A becomes engaged in apoptosis resistance, which has been confirmed in vitro, where a strikingly upregulation of cleaved caspase 9 and activated caspase 3 with unaltered caspase 8 activity has been seen in cisplatin-treated ASML-C4.4A^{kd} cells. Thus, it is the mitochondrial apoptosis pathway (Strasser *et al*., 2011) that is triggered in stressed ASML-C4.4A^{kd} cells.

Having experienced that C4.4A becomes engaged in motility and invasiveness via its association with α6β4, we speculated that the association with α6β4 may also contribute to apoptosis resistance. The hypothesis has been supported by our previous finding that in ASML^{wt} cells, apoptosis resistance, due to high level anti-apoptotic protein expression is triggered via CD44v6, but can be initiated via CD44, α6β4 or Met ligand binding. Activation of anti-apoptotic molecules downstream of CD44v6 mostly proceeded via activation of the MEK signaling cascade (Jung *et al*., 2011). However, preliminary experiments did not reveal differences in activation of the MEK signaling pathway between ASML^{wt} and ASML-C4.4A^{kd} cells. Instead there is evidence for strongly diminished activation of the PI3K/Akt pathway in ASML-C4.4A^{kd} cells. PI3K/Akt activation by β4 in cancer has been described by several groups (Giancotti, 2007; Nguyen *et al*., 2000; Shaw *et al*., 1997; Hintermann *et al*., 2001; Santoro *et al*., 2003; Trusolino *et al*., 2001), where α6β4 clustering by LN5 can be the initial trigger (Nguyen *et al*., 2000; Kippenberger *et al*., 2004). In line with the latter observation, the intracellular tail of β4 does not suffice for Akt activation. Also, it is unlikely that PI3K becomes directly activated by phosphorylated β4 (Merdek *et al*., 2007). Several pathways for β4 activation have been described. Besides activation via associated growth factors (Hintermann *et al*., 2001; Trusolino *et al*., 2001) or via insulin receptor substrate 1 and 2 (Kippenberger *et al*., 2004), raft recruitment and the interaction with raft-located tyrosine kinases are considered to provide the initial trigger (Giancotti, 2007). Our preliminary

data point towards raft recruitment as the most likely pathway according to the constitutive raft localization of C4.4A, the stimulation of α6β4 via LN5 and the recruitment of activated α6β4 via the joint LN5 ligand.

Thus, activation of anti-apoptotic molecules appears to be, at least, the dominating pathway of apoptosis resistance. Notably, C4.4A again acts as a linker without actively contributing to apoptosis resistance. Irrespective of this, strong downregulation of C4.4A has a striking effect on the loss of drug resistance *in vitro* as well as in *vivo*.

Taken together, a C4.4A^{kd} in a metastasizing tumor line confirmed our studies on C4.4A overexpression in a non-metastatic line (Rösel *et al*., 2002) as well as the *in vitro* analysis of C4.4A cooperativity-based actions. By its raft location, by binding to LN5, by associating with MMP14 and by recruiting α6β4 under stress conditions, C4.4A, though not essentially required for metastasis formation, plays an important role in tumor progression allowing tumor cells to disperse in the invaded organs and to become cytotoxic drug insensitive. Taking the selected steps in the metastatic cascade with a contribution of C4.4A, it appears of pathophysiological economy that C4.4A becomes selectively upregulated during wound repair and metastatic spread.

4.6 Conclusion

C4.4A is a metastasis-associated molecule expressed in several types of cancer with upregulated expression during tumor progression, as well as during tissue remodeling. Though being related to uPAR, there is no evidence for enzymatic activity of C4.4A and the function of C4.4A still remains elusive. The aim of my thesis was to shed some light on possible pathways of C4.4A activity.

C4.4A being frequently upregulated under hypoxic conditions, I speculated that HIF1α might trigger *C4.4A* transcription, particulary because the *C4.4A*

promoter contains HIF responsive elements. Though this has not been the case, I progressed during the first part of my thesis with evaluating C4.4A activity under hypoxic conditions, as hypoxia can well account for stress in a more general sense and hypoxia promoted C4.4A upregulation. These *in vitro* studies supported by an *in vivo* wound healing experiment revealed that C4.4A, which is a receptor for LN1 and LN5, exerts fuctional activity mostly via associating molecules, where α6β4 and MMP14 play a dominant role. By associating with activated α6β4, likely via the joint LN5 ligand, C4.4A promotes motility. Motility becomes strengthened by the association with MMP14, which has a strong impact on laminin degradation. It is particulary worthwhile noting that the raft located C4.4A-α6β4-MMP14 complex becomes internalized and is recovered in exosomes in a fuctionally active form, where the exosomal C4.4A-α6β4-MMP14 complex allows for matrix degradation even in the surrounding of C4.4A expressing epithelial cells. C4.4A also gets shed by TACE. But so far, possible functional activities of shed C4.4A have not been uncovered.

In the second part of my thesis, I controlled whether these features of C4.4A are of relevance for the metastasis process. To achieve this goal, I generated a C4.4A knock down of the highly metastatic ASML line. *In vitro* and *in vivo* experiments with this line confirmed the results of C4.4A activity under hypoxia. Thus, ASML-C4.4A^{kd} cells can metastasize but with significant delay and *in vitro* their migratory activity is strongly reduced. There is evidence that this a sequel of reduced α6β4 recruitment towards rafts and impaired MMP14 activation. The ASML-C4.4A^{kd} line provided evidence for a second major activity of C4.4A that has not been uncovered in hypoxia. C4.4A is engaged in apoptosis resistance. Our experiments exclude an involvement of C4.4A in receptor mediated apoptosis, but point towards C4.4A supporting anti-apoptotic molecule expression. This hypothesis remains to be experimentally proven, which includes our suggestion on a dominating role of the C4.4A-α6β4 complex.

Taken together, the failure to define the function of C4.4A, so far, likely is due to C4.4A predominantly acting as a linker molecule. Without question, α6β4 is the most important partner, where C4.4A will recruit activated (α6)β4 via their joint LN5 ligand, such that cell motility and apoptosis resistance signaling pathways become activated. The second important C4.4A partner is MMP14 that further strengthens motility of C4.4A expressing cells by LN degradation. Finally, I want to stress the point that even suggesting that C4.4A acts mostly as a linker, its "linker" activity is essential as motility, invasiveness and apoptosis resistance are severely impaired in ASML-C4.4A^{kd} cells.

C4.4A has a very restricted expression in non-transformed cells. Thus, I suggest that based on the presented studies, therapeutic interference with tumor progression could be possibly approached via a blockade of C4.4A.

5. SUMMARY

C4.4A was first identified in a screening designed to select metastasis-associated membrane proteins. Its expression is very restricted on normal tissue, but strongly upregulated during wound healing and progression in some tumors. The function of the molecule still remaining elusive, I aimed to shed some light on its transcriptional regulation and possible functions.

As wound repair and tumor growth are frequently associated with a state of hypoxia, it became tempting to speculate that hypoxia may promote *C4.4A* transcription. Moreover, the *C4.4A* promoter contains 3 HIF1α responsive elements (HRE), HIF1α being one of the most important transcription factors for hypoxia-regulated genes. However, *C4.4A* promotor activity was not altered after mutating the HRE or by co-transfection with HIF1α. Nevertheless, hypoxia is accompanied by an increase in C4.4A protein expression. Thus, hypoxia appeared a suitable model to evaluate functional activities of C4.4A, which I evaluated mostly in ASML cells, a metastasizing pancreatic adenocarcinoma line.

In vivo, C4.4A expression becomes strongly up-regulated in migrating keratinocytes during wound repair. *In vitro*, migration of C4.4A positive tumor cells on laminin 5 was strengthened under $CoCl_2$-treatment that served to mimic a hypoxic state. This relies on hypoxia-induced associations of C4.4A with α6β4 and MMP14. Migration-promoting activity of laminin 5 is further strengthened by its proteolytic processing via MMP14. Thus, the association of C4.4A with α6β4 and MMP14 allows for focalized laminin 5 fragmentation that promotes motility.

It is known that C4.4A can become shed, but also be recovered in exosomes. Both C4.4A shedding and exosomal release are stimulated under hypoxic stress. In line with the internalization of membrane microdomains, exosomes collected from $CoCl_2$-treated tumor cells contained the complex of C4.4A with α6β4 and

MMP14. Accordingly, the exosomal C4.4A-α6β4-MMP14 complex contributed to laminin 5 fragmentation, thus preparing a path for migrating epithelial and tumor cells.

To confirm the relevance of the association of C4.4A with α6β4 and MMP14 on metastasis formation, I generated a C4.4A knockdown of the highly metastatic ASML line. Distinct to ASML cells, ASML-C4.4A knockdown (ASML-C4.4A^{kd}) cells metastasize with a significant delay and do not invade surrounding tissue. The differences in the growth profile between ASML and ASML-C4.4A^{kd} cells could well rely on the association of C4.4A with MMP14 and, only under stress conditions, the recruitment of α6β4 towards the raft located C4.4A.

The ASML-C4.4A^{kd} cells revealed an additional activity of C4.4A that had not been observed before. ASML-C4.4A^{kd} cells were highly cisplatin susceptible, whereas there has been no evidence for reduced apoptosis resistance of ASML-C4.4A^{kd} in the absence of cisplatin. So far, I excluded receptor-mediated apoptosis induction, but the pathway accounting for the contribution of C4.4A to drug resistance remains to be explored.

Taken together, my studies confirmed the engagment of C4.4A in epithelial and tumor cell motility and invasiveness and provided for the first time a possible explanation by unravelling the linker function of C4.4A in recruiting α6β4 and activating MMP14. The novel finding of an engagement of C4.4A in drug resistance requires further exploration, that might open a pathway for therapeutically attacking metastases via C4.4A.

5. ZUSAMMENFASSUNG

C4.4A wurde bei einer Suche nach metastasierungsrelevanten Membranproteinen der Ratte entdeckt. Es zeichnet sich durch restringierte Expression im Normalgewebe aus, wird aber während der Wundheilung und bei einigen Tumoren, speziell während der Metastasierung, überexprimiert. Die Funktion von C4.4A ist weitgehend ungeklärt. Das Ziel meiner Arbeit war die Untersuchung der transkriptionellen Regulation und möglicher Funktionen. von C4.4A.

Da Wundheilung und Tumorwachstum in der Regel mit Hypoxie einhergehen, wurde zunächst die Möglichkeit einer Hypoxie-induzierten C4.4A-Transkription untersucht. Da der C4.4A-Promotorbereich drei HIF1α-Erkennungsmotive enthält und HIF1α einer der wichtigsten Transkriptionsfaktoren für Hypoxie-regulierte Gene ist, habe ich die Regulation der Transkription von C4.4A durch Hypoxie und HIF1α untersucht. Die C4.4A-Promotoraktivität änderte sich nicht durch die Einführung von Mutationen in die HIF1α-Erkennungsmotive oder durch Transfektion mit HIF1α. Dennoch wurde unter Hypoxie ein Anstieg der C4.4A-Proteinexpression beobachtet. Daher erschien Hypoxie ein gutes Modellsystem zur Evaluierung funktioneller Aktivitäten des C4.4A-Proteins. Ich habe dies bei der *in vivo* Wundheilung und hauptsächlich in der stark metatasierenden Ratten-Pankreasadenokarzinom-Zelllinie ASML untersucht.

Die C4.4A-Expression wird in migrierenden Keratinozyten während der Wundheilung i*n vivo* hochreguliert. *In vitro* wird die Migration von C4.4A-positiven Tumorzellen auf Laminin 5 unter $CoCl_2$-Behandlung, die zu einem Hypoxie-ähnlichen Zustand führt, verstärkt. Dies ließ sich auf eine Hypoxie-induzierte Assoziation von C4.4A mit dem Integrin α4β6 und MMP14 zurückführen. Die migrationsfördernde Aktivität von Laminin 5 wurde hierbei durch den proteolytischen Verdau von Laminin 5 mittels MMP14 verstärkt. Dies bedeutet, daß die Assoziation von C4.4A mit α4β6 und MMP14 lokal die Fragmentierung von Laminin 5 und damit Motilität fördert.

Es ist bekannt, daß die extrazellulären Domänen von C4.4A abgespalten werden können und C4.4A auf Exosomen angereichert ist. Sowohl das C4.4A-Shedding als auch die exosomale Freisetzung werden durch Hypoxie stimuliert. Auch der C4.4A-α4β6-MMP14-Komplex konnte auf Exosomen von $CoCl_2$-behandelten Zellen nachgewiesen werden. Entsprechend dem membranständigen Komplex, kann der exosomale C4.4A-α4β6-MMP14 Komplex Laminin 5 verdauen und somit die Migration von Epithelzellen und Tumorzellen unterstützen.

Um die *in vivo* Relevanz des C4.4A-α4β6-MMP14-Komplexes zu untersuchen, habe ich ASML-C4.4A^{kd}-Zellen generiert. Im Vergleich zu ASML-Zellen metastasieren ASML-C4.4A^{kd}-Zellen signifikant verzögert und dringen nicht in umgebendes Gewebe ein. Vorläufige Ergebnisse weisen daraufhin, daß die Unterschiede im Wachstumsverhalten der ASML-C4.4A^{kd}-Zellen auf die fehlende Assoziation von C4.4A mit MMP14 zurückzuführen sind. Hinzu kommt, daß auch unter Streßbedingungen die Rekrutierung von α4β6 in Rafts, in denen C4.4A und MMP14 lokalisiert sind, signifikant beeinträchtigt ist.

Darüber hinaus zeigen ASML-C4.4A^{kd}-Zellen eine weitere Besonderheit. Die Chemoresistenz von ASML-C4.4A^{kd}-Zellen ist im Vergleich zu ASML-Zellen deutlich reduziert. Eine Involvierung von C4.4A in Apoptoseresistenz war bisher nicht bekannt. Ich konnte rezeptorvermittelte Apoptoseinduktion ausschließen. Die zugrundeliegenden Signalwege müssen noch geklärt werden.

Meine Untersuchungen bestätigten die Beteiligung von C4.4A an der Motilität von Epithel- und Tumorzellen sowie der Invasivität von Tumorzellen. Sie liefern darüber hinaus erstmals eine mögliche Erklärung über die C4.4A-vermittelten Rekrutierung von α4β6 und die Aktivierung von MMP14. Erste Befunde zu C4.4A-vermittelter Chemoresistenz erfordern weitere Untersuchungen, die möglicherweise therapeutische Optionen eröffnen den Metastasierungsprozeß über C4.4A zu attackieren.

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7. ACKNOWLEDGMENT

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At last, I thank the Almighty God for his blessing.

8. LIST OF PUBLICATIONS

1. **Metastasis-associated membrane-bound and exosomal C4.4A promotes migration by associating with alpha6beta4 and MMP14 (in revision).**

Honoré Ngora[1,*], Uwe M. Galli[1,*,+], Kaoru Miyazaki[2], Margot Zöller[1,3]

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2. **The metastasis-associated molecule C4.4A promotes tissue invasion and anchorage independence (in preparation).**

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ABSTRACTS

1. Honoré Ngora, Uwe M. Galli and Margot Zöller. Transcriptional regulation of C4.4A under hypoxia. DKFZ PhD retreat , Weil Der Stadt, Germany ; 23- 25 june 2010.

2. Honoré Ngora, Uwe M. Galli and Margot Zöller. Hypoxia-promoted C4.4A expression: Consequences on keratinocyte and tumor cell migration by the association with alpha6beta4 and MT1-MMP. 5th International PhD Cancer Conference, Beatson Institute, Glasgow, Scotland; 15th- 17th June 2011.

9. CURRICULUM VITAE

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10. DECLARATION

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Erklärung

Hiermit erkläre ich, dass ich keine vorausgegangenen oder laufenden Promotionsversuche unternommen habe.

12.09.2011