International Journal of Molecular Biology & Biochemistry.
Volume 1, Number 1 (2013), pp. 39-56

http://www.irphouse.com

ADAM10 is the Responsible Sheddase of the CD44 Ectodomain in Human Melanoma Cells

Christian Haiduk[1], Thea Eichenberg[1], Christian Ihling[2], Sven Rothemund[3], Miroslav Malesevic[4], Andrea Sinz[2], Jan C. Simon[1*], Ulf Anderegg[1#*]

[1] Department for Dermatology, Venereology and Allergology, University Hospital Leipzig, Germany
[2] Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Germany
[3] Interdisciplinary Center for Clinical Research, University of Leipzig, Germany
[4] Max Planck Research Unit for Enzymology of Protein Folding, Germany
[*]both authors contributed equally
[#]corresponding author: Ulf Anderegg
Department for Dermatology, Venereology and Allergology
University of Leipzig, Medical Faculty
Johannisallee 30 04103 Leipzig
phone: +49-341-9725881 fax: +49 341 -9725878
e-mail: Ulf.Anderegg@medizin.uni-leipzig.de

Abstract

The liberation of CD44s ectodomain plays an important functional crucial role in the tumor biology of human malignant melanoma (MM) cells. It has been shown that the shedded CD44 ectodomain suppresses the proliferation induced by hyaluronan in human MM cells and that ADAM10 is essential for CD44s shedding. Here we provide evidence that ADAM10 indeed cleaves CD44s directly. Co-transfection of CHO-cells with CD44 and ADAM10 resulted in strong CD44 ectodomain release. Both, native and recombinant CD44, were cleaved at the identical cleavage site discovered by us. Furthermore, this study provides an putative binding site of ADAM10 for CD44s for the first time. Using a peptide library of ADAM10-derived peptides we could identify four main peptide motives in the disintegrin and cysteine-rich domains of ADAM10 which bind soluble CD44 ectodomain. Moreover, one of these peptides can interfere with the CD44 shedding in MM cells. The identification of ADAM10 as sheddase of CD44s and the characterization of its shedding mechanism in human MM cells could be a possible target for the development of new therapeutic strategies.

Keywords: malignant melanoma, CD44 cleavage, ADAM10, protein interaction, restriction binding site

Abbreviations:

ADAM: a disintegrin and metalloproteinase; CD44s: standard isoform of CD44; HA: hyaluronan; MT1-MMP: type I-transmembrane matrix metalloproteinase (MMP14); solCD44: soluble form of CD44-ectodomain after shedding; $ADAM10_{D+C}$: the disintegrin and cysteine-rich domains of ADAM10, MM: malignant melanoma, DPA: Dpa: N-3-(2, 4-Dinitrophenyl)-L-2,3-diaminopropionyl MCA: Mca: (7-Methoxycoumarin-4-yl)acetyl

Introduction

CD44 glycoproteins belong to a family of cell surface receptors with a wide structural variety of isoforms, which are generated by alternative splicing and different glycosylation. Being a type I protein, all CD44 isoforms possess an intracellular, a transmembrane and an extracellular domain. The extracellular domain can be subdivided into a globular link domain binding the CD44 major ligand hyaluronan (HA) and a highly glycosylated and variable stalk region, which is located proximal to the membrane and comprises the restriction sites of CD44. Differing sizes of the stalk region due to the expression of variable exons allows a subdivision of the CD44 splicing variants (referred to as CD44v) (reviewed by [1]. The interaction of CD44 and HA regulates the proliferation, migration and differentiation of benign and malignant cells [1-6]. For example, silencing of CD44 in malignant pleura mesothelioma cells diminished the HA induced proliferation and invasion significantly [7]. Blocking of CD44-HA interaction with function blocking antibodies reduced adhesion, motility and invasion in human breast cancer cells suggesting antibodies against specific CD44 epitopes as therapeutics [8]. However, the underlying mechanisms for the function of CD44 are still objects of intensive research. The CD44 within the cell membrane could either act as an organizing platform for other proteins or interact with growth factors and growth factor receptors [9]. The interaction of the CD44 intracellular domain with ezrin or the tumor suppressor merlin has been shown to function as an alterable switch between cell proliferation and apoptosis in a cell density dependent manner [5].

The standard CD44 isoform (referred as CD44s) is 85-100kDa in size and lacks the epitopes encoded by the variant exons. The widely expressed CD44s binds strongly to HA [10] and is most frequently described in the context of several cancers (reviewed by [11]. In human melanoma cells, the interaction of CD44s with its ligand HA promotes the tumour cell proliferation and metastatic formation. In addition, CD44s is strongly upregulated in melanoma indicating its important role in melanoma progression [2,12].

In cancer research, the process of ectodomain shedding has reached significance since it has been shown to enhance the detachment and migration of tumour cells [13,14]. Moreover, it also modifies intercellular communication of tumour cells with

stromal cells and alters signalling from and to the extracellular matrix [14]. Shedding is described as the cleavage of membrane proteins by specific proteases followed by the release of the shed ectodomain into the intercellular space where this ectodomain can compete for ligand binding. CD44s can be shed by a protease resulting in a soluble full functional ectodomain (solCD44). High serum levels of solCD44s correlate with a higher metastatic potential and a poor prognosis in colorectal cancer and lymphoma [15,16]. On cellular level shedding of the CD44-ectodomain plays a critical role in tumour cell migration [4,17]. On the other hand, in human melanoma solCD44s prevents the HA driven cell proliferation probably by competition with the membrane bound CD44s [3].

Several studies suggested different candidate sheddases to be involved in the cleaving of the CD44s ectodomain, mainly the type I-transmembrane matrix metalloproteinase (MT1-MMP), and the a disintegrin and metalloproteinase proteins 10 and 17 (ADAM10, ADAM17) [17-23]. Using a recombinant Tet-off system in the melanoma cell line A375, Nakamura and colleagues could induce a CD44s cleavage by the MT1-MMP [21]. The corresponding cleavage site at Gly^{192} was identified and resulted in a 37kDa fragment of soluble CD44. However, the constitutive shedding of CD44s in melanoma cells results in a liberated CD44s ectodomain of 65-70 kDa in size and origins from the cleavage at Ser^{249} [21] by an other protease than MT1-MMP. Thus, ADAMs were alternatively suggested to regulate the constitutive CD44s shedding [17,21]. Murai et al. demonstrated that downregulation of ADAM10 by RNA interference resulted in a suppression of CD44s cleavage in human glioblastoma cell line U251MG [20]. Subsequently, also ADAM17 was shown to induce proteolysis of CD44s [17]. Our group could show that ADAM10, but neither ADAM17 nor MT1-MMP is involved in the constitutive shedding of CD44s in several human melanoma cell lines. After blocking of ADAM10, ADAM17 and MT1-MMP by appropriate siRNAs, only silencing of the ADAM10 mRNA expression resulted in a significant reduction of CD44s shedding. Specific inhibitors blocking the activity of ADAM10 or ADAM10 and ADAM17 confirmed the involvement of ADAM10 in CD44s shedding. As a consequence, decreasing solCD44s by ADAM10 knockdown in MM cells significantly induced cell proliferation [18]. However, having identified ADAM10 as the crucial enzyme for the constitutive shedding of CD44s, the molecular mode of action such as interaction pattern, exact cleavage site and binding motifs had not been identified. In the present study we describe the cleavage site in the CD44s protein in human melanoma and show that ADAM10 is able to cleave these sequence motives. Furthermore, we demonstrate that ADAM10 directly interacts with CD44s and we could identify four putative binding sites in the ADAM10 disintegrin and cysteine-rich domain, which have the ability to bind CD44s.

Results

Coexpression of ADAM10 and CD44s in CHO cells leads to the liberation of solCD44s

The shedding of the CD44s ectodomain in the extracellular space can be measured by

analyzing the amount of solCD44s in the supernatant of cells, which can be determined by ELISA. Almost all melanoma cell lines shed CD44s albeit in markedly different amounts. In high-shedding melanoma cell lines like HT144, MV3, and Bro the concentration of solCD44s may exceed 120ng/10^6 cells in 24 hours. Whether a direct interaction of ADAM10 and CD44s may initiate the cleavage of the CD44s ectodomain or if other proteins are required still remains unclear. To address this issue, we cotransfected these two proteins in CHO cells, which resulted in a CD44s ectodomain release comparable to that of the high shedding melanoma cell line Bro (Fig.1). The shedding capacity of the cotransfected CHO cells was in a range of 80 ng/10^6 cells in 24 hours. Transfection of either ADAM10 or CD44s alone resulted in a very weak or no detectable release of the CD44s ectodomain (Fig.1a). Furthermore, we compared the molecular weights of the shedded ectodomains from cotransfected CHO cells and the melanoma cell line *Bro* by western blot analysis. The CHO cell-derived ectodomain of CD44s displayed the same mobility like that of the melanoma cells (Fig. 1b) implicating that the CD44s ectodomain shed by cotransfected CHO cells has the same glycosylation pattern and the same restriction site as the melanoma cell derived-CD44s ectodomain.

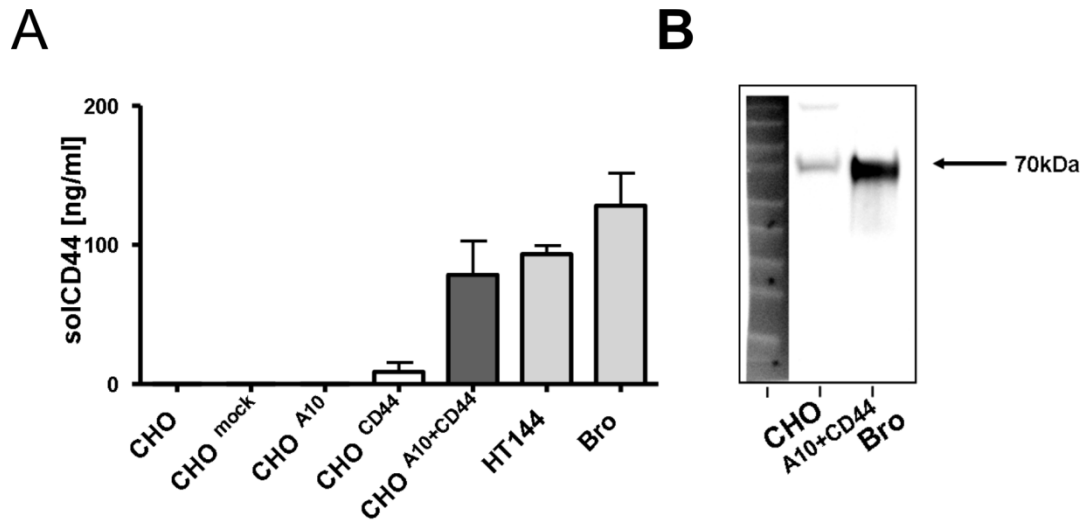


Figure 1: (*A) Detection of soluble CD44s in supernatants of transfected CHO cells.* CHO cells were transfected with ADAM10 (A10), CD44s or both proteins (A10+CD44); empty vector transfected (mock) and non transfected CHO cells served as negative, *HT144* and *Bro* cell lines (CD44s high shedding cell lines) as positive controls. Supernatants were harvested after 24h culture in serum-free medium and ELISA was performed to measure CD44s content. Data are mean (±SD) of three to five different experiments. **$p<0.05$ (Mann-Whitney-test). *(B) Soluble CD44s from transfected CHO cells display the similar molecular weight and glycosylation pattern compared to soluble CD44s from Bro melanoma cells.* Immunoblot of soluble CD44s derived from supernatants from transfected CHO cells and *Bro* melanoma cell line was performed.

CD44s ectodomain is cleaved by ADAM10 at Ser^{249}

Next. we determined the exact cleavage sites of the CD44s ectodomain in *Bro* melanoma cells and in CD44/ADAM10 cotransfected CHO cells. The shed ectodomain was purified from the supernatants, proteolyzed with AspN. Thereafter, the obtained fragments were deglycosylated with PNGaseF and further analysed by nano-HPLC / MALDI-TOF/TOF mass spectrometry. To determine the CD44s restriction site the very c-terminal fragment of the digested ectodomain had to be identified. Here, we could identify a 2418 Da fragment derived from cotransfected CHO cells as well as from the melanoma cell line *Bro* that comprises the amino acids (AA) Asp^{226} to Ser^{249} (Fig. 2). Because Ser^{249} is flanked by Glu^{250} in the CD44s ectodomain this cleavage site does not result from the AspN digestion. Thus we provide evidence that ADAM10 cleave CD44s at Ser^{249}.

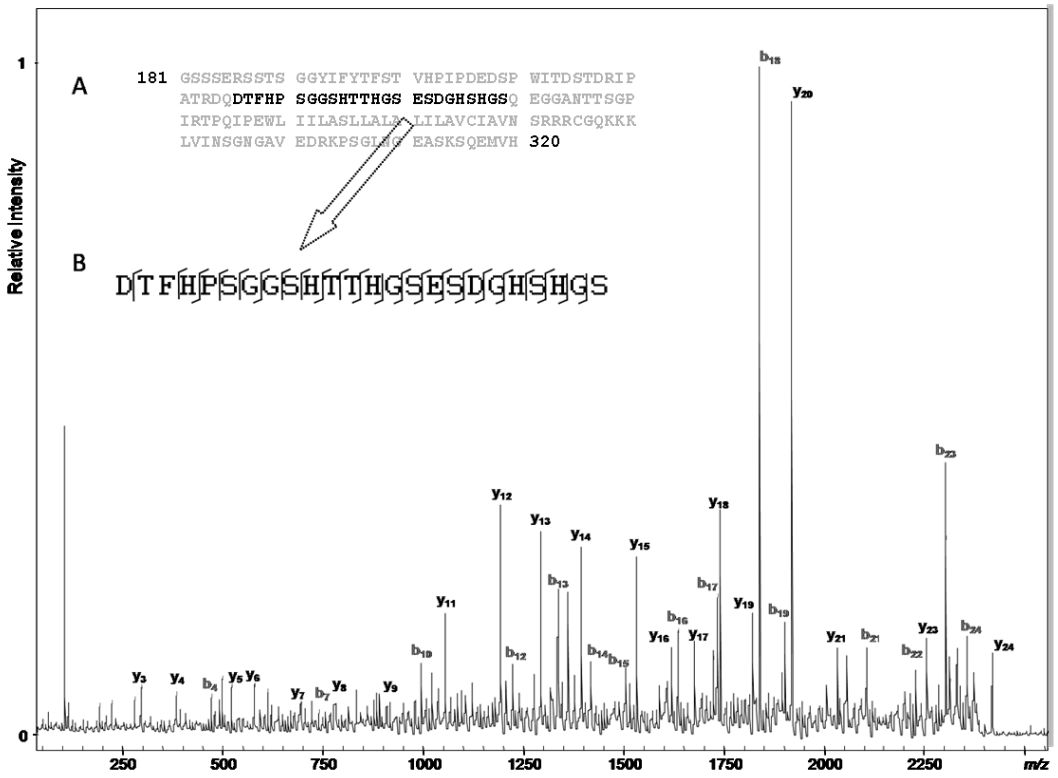


Figure 2: *Identification of the C-terminal peptide of the shed CD44s ectodomain.* MALDI-TOF/TOF fragment ion mass spectrum of the C-terminal peptide of the shed CD44s ectodomain derived from *Bro* cells. After AspN digestion and deglycosylation the C-terminal peptide of the shed CD44s ectodomain was identified based on MS/MS data (B). The c-terminal peptide is highlighted in the C-terminal sequence of full length CD44s antigen (A).

Mca-Dpa-TNFαAV and Mca-Dpa-TNFαAVΔSQ cleavage by recombinant human ADAM10

To confirm that ADAM10 is capable to cleave CD44s at Ser^{249} in vitro peptide digests were performed. Fluorogenic peptides according to the sequence of TNFα, a known substrate of ADAM10, were synthesized. The Mca-Dpa-TNFαAV peptide consists of a restriction site between an Ala and Val residue, which is flanked n-terminally by the fluorescent Mca group and c-terminally by the quenching Dpa group. After peptide cleavage an increased fluorescent signal was observed due to the loss of internal quenching and measured in real-time using a spectrofluorometer. For validation of ADAM10s aptitude for cleaving a substrate at Ser249 we modified the TNFα restriction site Mca-Dpa-TNFα*AV* to the CD44s restriction site Mca-Dpa-TNFα*AVΔSQ*. As control a scrambled sequence in Mca-Dpa-TNFαAVΔ*NI* was used. The hydrolysis of 10uM Mca-Dpa-TNFα*AV* and Mca-Dpa-TNFα*AVΔSQ* by 160nM rhADAM10 led to a three-fold increase of the fluorescent signal compared to the scrambled control Mca-Dpa-TNFαAVΔNI over a period of 85 minutes (Fig 3). The reaction curve was nearly linear over the time and without the enzyme no fluorescent signal was observed. These data implicate that ADAM10 is able to cleave a substrate at CD44s restriction site with the same efficiency as a substrate comprising the TNFα restriction site.

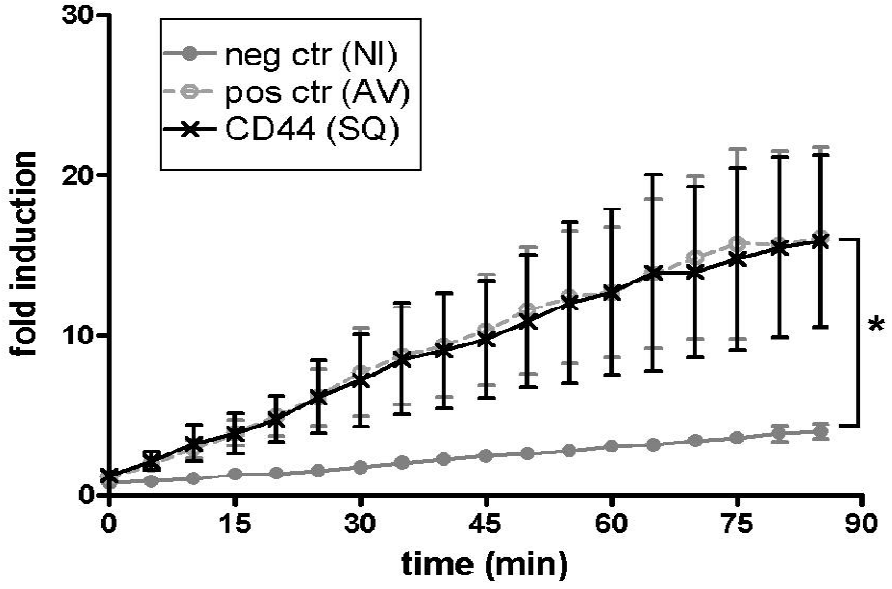
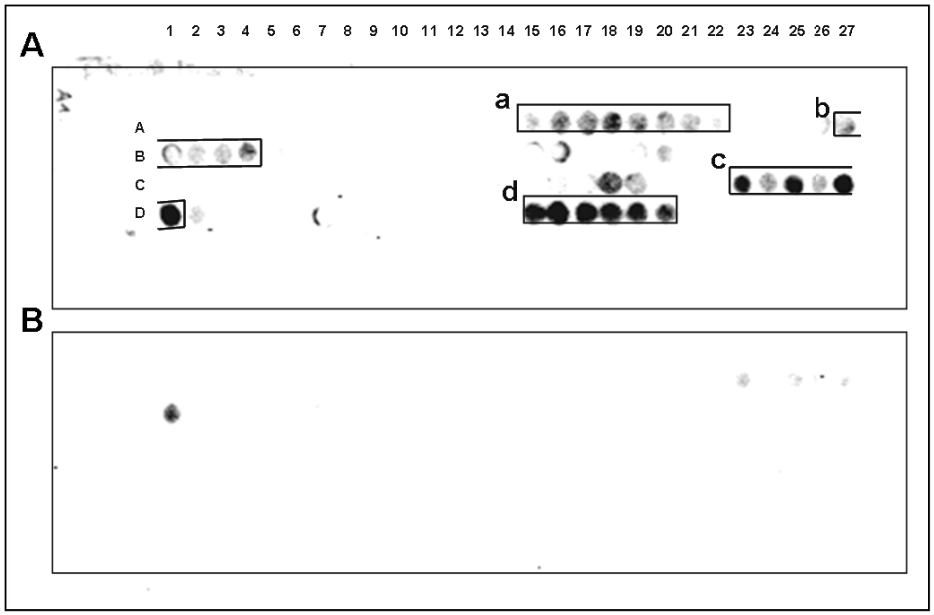


Figure 3: *Recombinant human ADAM10 cleaves a peptide at the CD44s restriction site.* Fluorogenic peptides with a flanking sequence according to the ADAM10-cleavage site in huTNFα and consisting of either the TNFα cleavage motif (Mca-Dpa-TNFαAV) or the CD44s cleavage site (Mca-Dpa-TNFαAVΔSQ) were digested by rhADAM10. As negative control a peptide was used consisting of Asn-Ile at the cleavage site. All data are mean (±SD) of three different experiments. *$p<0.05$ (un-paired t-test)

Four putative binding sites in ADAM10s disintegrin and cysteine-rich domain bind CD44s

The disintegrin and cysteine-rich domains of ADAMs have been reported to be essential for substrate recognition and binding. (reviewed by [24]) To identify the putative binding sites of ADAM10 we performed a peptide library scan spanning the sequence of ADAM10 from Gln^{457} – Glu^{672}, which include in part the metalloprotease domain and the complete disintegrin and cysteine-rich domains. Each peptide corresponds to 12 AA of the ADAM10 sequence with a forward shift of two AA. Purified CD44s bound to four clusters of peptides (Fig. 4A), which correspond to the ADAM10 motives Phe^{485}-Ser^{528}, Ala^{541}-Gln^{560}, Lys^{600}-Cys^{632} and Leu^{647}-Leu^{666}. The first two motives are located in the disintegrin domain of ADAM10 while the other two represent the binding capacity of the cysteine-rich domain. As control the same a peptide array was performed without solCD44s incubation resulting in no signal detection (Fig. 4B). In consideration of the peptide overlap on the array, we narrowed down the motives to four minimal binding motives comprising the AA displayed in Fig. 4C.



C

Stretch	Spot	according amino acid sequence	putative minimal binding motif
a	A15-A22	FDANQPEGRKCKLKPGKQCSPSQGPC	CKANQPEGRKCKLKPG
b	A27-B4	PCCTAQCAFKSKSEKCRDDS	CAFKSKSE
c	C23-D1	TGSVQWSRHFSGRTITLQPGSP	SRHFSGRTIT
d	D15-D20	VDADGPLARLKKAIFSPELYEN	LARLKKAIFS

Figure 4: *CD44s ectodomain binds to distinct stretches of an ADAM10 peptide library array.* (A) An array of overlapping 12mer peptides according to the

disintegrin and cysteine-rich domain of ADAM10 was synthesized with a forward shift of two amino acids (AA). The solCD44s protein purified from melanoma cell supernatants interacts with four distinct peptide stretches (a-d). (B) The array was performed in the same way as in A without CD44s. (C) The corresponding peptide sequences and the putative minimal binding motives are depicted.

ADAM10-derived peptide can impair CD44s shedding from Bro and HT144 melanoma cell lines

Next we aimed to test whether the binding of the four peptides representing putative binding motives of ADAM10 has a functional impact on CD44s shedding in melanoma cells. The four derived peptides (Tab.1) were added to the cell cultures of the high shedding melanoma cell lines Bro and HT144 in a 10 µM concentration. While the peptides comprising the motifs one to three had no effect, the very c-terminal binding motif (pep4) led to a significant decrease in CD44s ectodomain liberation (Fig 5A,B) in both cell lines. The inhibitory effect of pep4 occurs in a dose dependent manner (Fig 5C). This data indicate that at least one of the four peptides is able to compete with the interaction of ADAM10 and membrane bound CD44s.

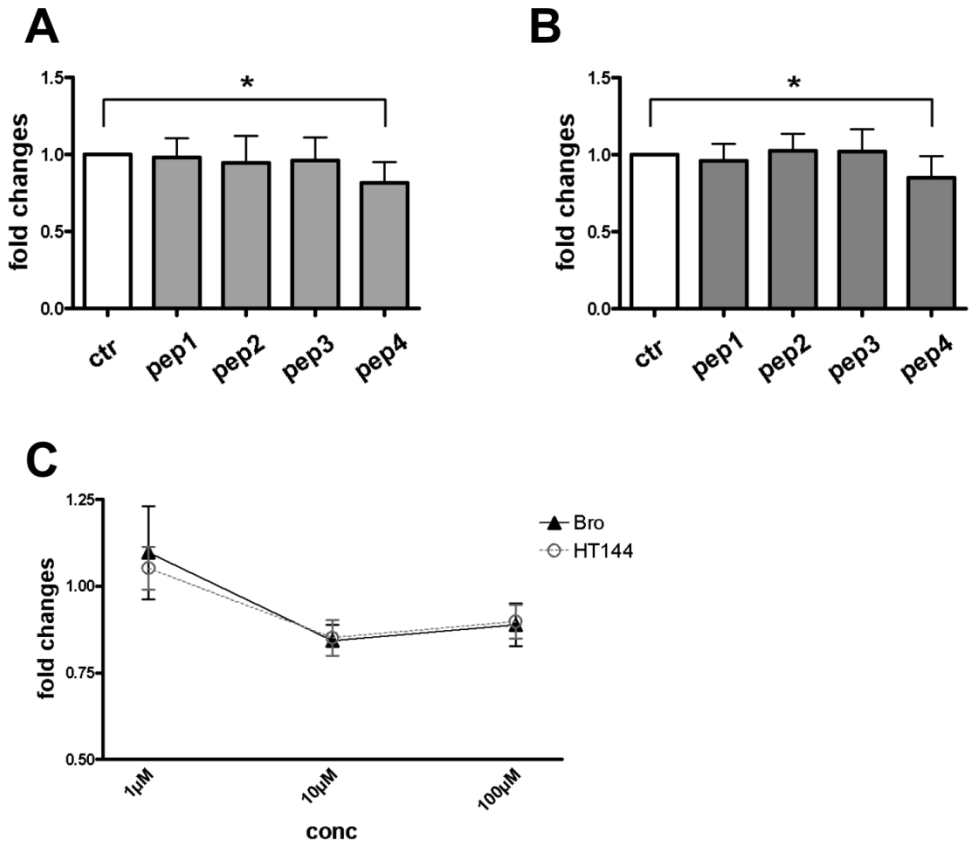


Figure 5: *Peptides comprising the putative binding sites of ADAM10 affect CD44s ectodomain shedding in melamoma cells.* The high shedding melanoma cell lines *Bro* (A) and *HT144* (B) were cultured in FCS free medium consisting of 7.5 µM of a

peptide which correlates with one of the new identified minimal binding sites of ADAM10 or a mixture of all of them (peptide1-4, Tab.1). After 24 hours the cell free supernatant was collected to measure the concentration of the shed CD44s ectodomain. The mean and standard deviation of seven independent experiments is represented. P values (= 0.0131 for *Bro* cells and 0.0279 for *HT144*) were calculated using paired t-test. (C) The inhibition of CD44 shedding by peptide 4 is dose-dependent in both MM cell lines. n=3-7

Discussion

Ectodomain shedding plays a fundamental role in many cellular functions during development, inflammation and cancer [25-27]. The process of extracellular domain cleavage of membrane spanning proteins is often performed by metalloproteinases such as ADAM9, ADAM10 and ADAM17. These ADAMs have been described to be involved in shedding of various proteins including cytokines, growth factors, receptors, adhesion proteins thereby influencing cell-cell adhesion, signalling, differentiation and proliferation [28-30]. The involvement of ADAMs in human melanoma development, progression and metastasis has been described in several studies [18,31,32]. In a previous study, our group identified ADAM10 as the key protease for the constitutive ectodomain shedding of CD44s in human melanoma cell lines [18]. Blocking ADAM10 activity or expression inhibited the CD44s shedding and boosted the proliferation of melanoma cells which was demonstrated to be Ca^{++} dependent [33]. These findings consider ADAM10 over-expression or activation as a potential therapeutic option for the treatment of primary MM. On the other hand, Lee and colleagues demonstrated that high levels of ADAM10 and shedding of the substrate L1-CAM are correlated with an induction of MM cell proliferation and migration in melanoma metastases [31]. Therefore, specific regulatory mechanisms of intended ADAM10-mediated effects have to be identified based on a deeper understanding of ADAM10 function and mechanisms in primary MM.

Here we aimed to show whether CD44s is a direct substrate of ADAM10 and to identify the domains of ADAM10, which are responsible for CD44s substrate binding and cleavage. We demonstrate for the first time, that ADAM10 directly interacts with CD44s and cleaves it at Ser^{249}. Additionally, we identified four putative binding sites for CD44s in the disintegrin and cysteine-rich domain of ADAM10.

First, we examined the ability of ADAM10 to cleave CD44s. Cotransfection of ADAM10 and CD44s into CHO cells resulted in the release of the CD44s ectodomain at levels similar to the melanoma cell lines HT144 and Bro. Since no solCD44s was detected in CHO cells transfected with ADAM10 or CD44s alone, a direct interaction between ADAM10 and CD44s with a direct cleavage of CD44s by ADAM10 was conceivable. To verify this, pull down assays using either CD44 or ADAM10 as bait were performed. However, these experiments were not successful. This could be due to the supposed very short lift interaction of a protease and its substrate. Furthermore, a native surrounding membrane structure might be essential for the proposed interaction of ADAM10 and CD44 and the release during cell lysis could resolve this interaction. Thus, we performed a bimolecular fluorescence complementation

approach (BiFC) in which positive signals accumulate over the time in contrast to a pull down assay. We show that fluorescence levels of CHO cells co-transfected with CD44 and ADAM10 were comparable to a positive control (Adiponectin-receptor-domain interaction) (Figure S1). The identification of the direct CD44s/ADAM10 interaction *in vitro* is not thoroughly transferable into an *in vivo* situation since for example the molecular ratio or the compartment expression could be incisively altered *in vitro*. Additionally, other proteins could be associated to CD44s or ADAM10 *in vivo* and regulate the shedding process of CD44s in melanoma cells. In summary, the co-transfection experiments with the subsequent purification of the shedded CD44s ectomain and the identification of the native cleavage site regardless of the source suggest that ADAM10 is able to shed CD44s directly. The identified cleavage site at Ser^{249} is in agreement with a previous study, where Nakamura and colleagues identified Ser^{249} beside other cleavage sites Gly^{192} and Gly^{233} in human melanoma cell line A375. In the study of Nakamura, the metalloproteinase MT1-MMP had been suggested to be responsible for the cleavage sites Gly^{192} and Gly^{233} [21]. However, the responsible sheddase for the cleavage site Ser^{249} remained elusive in their study. Here, together with our previous work [18] we provide conclusive evidence for CD44s cleavage by ADAM10 at Ser^{249} in human melanoma cells. To verify the restriction site of CD44, an *in vitro* digestion of the purified full length CD44 protein by recombinant ADAM10 was carried out. Unfortunately, in our hands an *in vitro* digestion was not successfull. We assume that this might be due to an altered protein folding of CD44 after isolation from cell membrane. Since ADAM10 cleaves native CD44 close to the membrane it is possible that a perturbed folding disturbs the process of shedding irreversibly. Since a previous report described that the substrate specificities of ADAM10 and ADAM17 depend in part on the sequence of the cleavage site and the surrounding residues [34] we tried to verify the cleavage site at Ser^{249}. Using a biochemical peptide cleavage assay we could show that the recombinant, functional ADAM10 ectodomain is able to cleave a peptide at the CD44s restriction site comparable to the TNFα restriction site. This is in accordance with Caescu et al. [34] who found that a Serin residue in the P1 and a Glutamine residue in the P1´position is a motif for an ADAM10 substrate. In addition to the cleavage motif in the active centre of the enzyme the disintegrine and cysteine-rich domains of ADAMs have been reported to be responsible for the substrate recognition [35-37]. A peptide library spanning the sequence of the disintegrine and cysteine-rich domain of ADAM10 ($ADAM10_{D+C}$) revealed four putative motifs in the $ADAM10_{D+C}$ region. The first two motifs are located in the disintegrin domain and comprise the residues Cys^{485}-Gly^{500} (ADAM10dCys^{485}-Gly^{500}) and Cys^{515}-Glu^{522} (ADAM10d Cys^{515}-Glu^{522}) whereas the motives three and four in the cysteine-rich domain include the residues Ser^{616}-Thr^{625} (ADAM10c Ser^{616}-Thr^{625}) and Leu^{654}-Ser^{663} (ADAM10c Leu^{654}-Ser^{663}). In 2005, Janes et al., could show that the substrate recognition of ephrin-A5 is mediated by an acidic pocket in the cysteine-rich domain [38]. Although putative binding motives identified in our experiments are apart from this mentioned acidic binding pocket, at this state we do not exclude that ADAM10 binds CD44s by this way too. A peptide library scan considers only linear epitopes but not possible three dimensional structures. However, in human melanoma cells the binding motif

ADAM10cLeu^{654}-Ser^{663} seems to play a considerable role in the recognition and/or binding of CD44s since a peptide composed of the ADAM10cLeu^{654}-Ser^{663} probably competes with the according binding motif of ADAM10 resulting in a significant down regulation of shedded solCD44s in melanoma cells. Peptides consisting of the other putative binding motifs identified by us did not affect the solCD44s release. It is possible that the different binding motifs possess a high redundancy in substrate recognition and binding.

This functional impact of an ADAM10-derived peptide on CD44s-shedding might be due to competitive binding to the CD44s target region and supports experimental findings from the peptide array. To the best of our knowledge, this is the first study showing a sequence motif of ADAM10 which significantly influences the CD44s shedding capacity of ADAM10. Further *in vivo* experiments have to be performed to verify that this sequence influences biological effects that are related to CD44s shedding like proliferation, differentiation and metastasis of malignant melanoma.

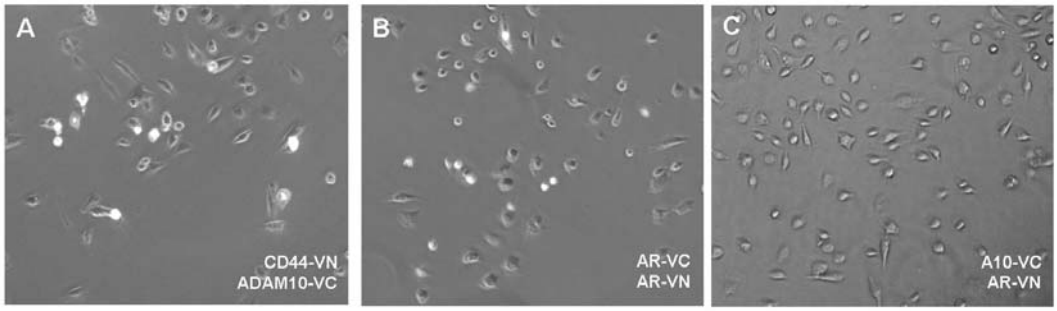


Figure S1: *BiFC signal detection in CD44s and ADAM10 co-transfected CHO cells* CHO cells were co-transfected with cDNAs for huCD44s, bovADAM10 and Adiponectin-Receptor1 (AdipoR1) coupled to either the c-terminal domain (VC) or the n-terminal domain of the Venus protein (VN) as described elsewhere (Kosel et al., 2010). Interaction of these two co-transfected proteins results in BiFC signals from interacting domains VC and VN detectable 36 hours after transfection. (A) CHO co-transfected cells with CD44s-VC and ADAM10-VN revealed distinct fluorescence signals. (B) Similar signals were detectable in CHO cells transfected with AdipoR1-VN and AdipR1-VC which are able to form a homo-dimer (Kosel et al., 2010). (C) co-transfection of ADAM10-VN and AdipoR1-VC did not result in any signals. These data indicate a specific interaction of ADAM10 and CD44s. One representative example out of n=5 is shown. Kosel D, Heiker JT, Juhl C, Wottawah CM, Blüher M, Mörl K, Beck-Sickinger AG. Dimerization of adiponectin receptor 1 is inhibited by adiponectin. 2010 J Cell Sci.;*123*:1320-8.

Conclusion

This study demonstrates that the proteinase ADAM10 cleaves the ectodomain of CD44s in a direct interaction manner in human melanoma cells. Furthermore, Ser^{249} was identified as the cleavage site in ADAM10-mediated shedding. Moreover, the

ADAM10-derived peptide sequence cLeu^{654}-Ser^{663} was shown to significantly reduce CD44s shedding by melanoma cells suggesting that this sequence is an important binding motif of ADAM10 during CD44s-ectodomain shedding

Materials and Methods

Materials

The following antibodies were used: mouse anti-ADAM10 (163003, R&D Systems, Minneapolis, USA), mouse anti-CD44 (SFF-304, BenderMedsystems, Vienna, Austria) and polyclonal rabbit anti-mouse peroxidase IgG (Sigma, Hamburg, Germany).

All Peptides used in this study were synthesised following the standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid phase peptide synthesis strategy and are displayed in Table 1.

Table 1: ADAM10-derived peptides used for blocking experiments of CD44s shedding in melanoma cell lines

abbreviation	minimal binding motif	peptide sequence
pep1	ADAM10dCys^{485}-Gly^{500}	CKANQPEGRKCKLKPG
pep2	ADAM10dCys^{515}-Gly^{522}	CAFKSKSE
pep3	ADAM10cSer^{616}-Thr^{625}	SRHFSGRTIT
pep4	ADAM10cLeu^{654}-Ser^{663}	LARLKKAIFS

Cell culture

The melanoma cell lines *Bro* (kindly provided by Dr. J. Eberle, Berlin, [39], *HT144* (kindly provided by Dr. van Muijen, [40] and CHO K1 cells (LGC Promochem Wesel, Germany) were cultured in RPMI or DMEM medium (Biochrom, Berlin, Germany), respectively, supplemented with 10% fetal calf serum (FCS, PAA, Pasching, Austria) and maintained at 37°C in a humidified 5% CO_2 atmosphere. For transfection, CHO cells were grown in six well plates until they reached approximately 50% confluence. Subsequently, transfected CHO cells were cultured in DMEM/FCS medium additionally supplemented with G418 (500 µg/ml) or Blasticidin S (8 µg/ml, InvivoGen, San Diego, California). All cultures were passaged by detaching the cells with trypsin/EDTA solution (Biochrom, Berlin, Germany).

Isolation of solCD44s

Soluble CD44s was collected from cell culture supernatants after 48h of serum starvation. Supernatants were cleared thereafter by centrifugation and filtration. Further purification was achieved by. anion exchange chromatography (aIEX) followed by hydrophobic interaction chromatography (HIC). Both aIEX and HIC were performed using an Äkta Purifier system (GE Healthcare, Munich, Germany). The purified solCD44s was dialysed and concentrated using Vivaspin® 15 Centrifugal Concentrators (Vivaproducts, Littleton, MA, USA). The purity of

solCD44s was monitored by Coomassie stained SDS PAGE. Amounts of solCD44s were quantified using ELISA for CD44 detection (BenderMedsystems, Vienna, Austria).

In-solution digestion and LC/MS/MS analysis

Samples of solCD44s derived from transfected CHO cells or cell line *Bro* were digested overnight using endoproteinase AspN (Roche, Mannheim, Germany) according to the manufacturer's protocols. To facilitate mass spectrometric identification, peptides were deglycosylated with PNGase F (Roche). Samples were analysed by nano-HPLC/MALDI-TOF/TOF mass spectrometry (Ultimate 3000 nano-HPLC system, Dionex and Ultraflex III, Bruker Daltonik, Leipzig, Germany) or nano-HPLC/nano-ESI - LTQ-Orbitrap - MS/MS (Ultimate nano-HPLC system, Dionex and LTQ-Orbitrap XL, ThermoFisher Scientific, Schwerte, Germany) for solCD44s from *Bro* cells or CHO cells, respectively. Experimental setups for LC/MS/MS analysis were used as described elsewhere [41]. Data were searched against an in-house database using Mascot (Mascotserver 2.2, Matrixscience, London, England) and peptides were identified based on MS/MS data.

Peptide digestion

The Mca-Dpa-TNFαAV, Mca-Dpa-TNFαAVΔSQ and Mca-Dpa-TNFαAVΔNI peptides, consisting of the TNFα restriction site (AV), the CD44s restriction site (SQ) or no restriction site (NI) were cleaved with recombinant human ADAM10 (rhADAM10, R&D Systems) according to the manufacturers protocol. Briefly, 10 µg of each peptide were dissolved in 95 µl reaction buffer (25 mM Tris, 2,5µM ZnCl2, 0,005% Brij 35, pH 9) and supplied in a 96 well Special Optic Plate (Corning B.V., Amsterdam, Netherlands). Subsequently, 500 ng rhADAM10 in 5µl reaction buffer was added. The cleavage of the peptides was measured at 37 °C all 5 minutes over a period of at least 85 minutes in a Synergy HT Multi-detection reader (Bio-tek, Bad Friedrichshall, Germany). The emission and excitation filters used were 320/20 and 400/30.

CHO cell transfection and analysis of CD44s-shedding

Human recombinant full-length sCD44s was cloned from a cDNA of *Bro* cells into pcDNA6.1 expression vector (Clontech, Heidelberg, Germany). Bovine recombinant ADAM10 in piresNeo expression vector was a kind gift from Dr. E. Parkin (Lancaster, UK; [42]. For transfection, 0.25µg plasmid was transfected into CHO cells using LipofectamineTM 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection the growth medium was replaced and transfected cells were selected over a period of 10 days by application of G418/ml or Blasticidin S medium. To measure the shedding capacity of the transfected CHO cells, 2 x 10^5 cells/well were seeded in six well plates. After 12h cells were washed twice with PBS and the medium changed to 1ml minimal medium without FCS. The concentration of solCD44s was determined in supernatants collected after 24h by ELISA.

Peptide array
The peptide array was synthesised according to the standard SPOT synthesis protocol as described elsewhere [43]. The following peptide array scan was performed according to Frank and Dübel [44]. In brief, membranes were washed once in methanol and three times in 20mM HEPES buffer (HB, pH 7.4) and incubated in blocking buffer (BB, 5% dry milk in HB) overnight. Subsequently, membranes were incubated with either 10µg/ml solCD44s or 10µg/ml bovine serum albumin (BSA, Sigma, Munich, Germany) in BB for three hours. After washing the membranes three times with HB, they were then incubated with anti-CD44 antibody (4µg/ml in BB) for additional 3h. Repeated washing steps followed before incubating the membranes with HRP-conjugated goat-anti-mouse detection antibody in BB. After 2h of incubation the detection antibody was washed out three times with HB and the bound solCD44s visualized with ECL solution (Pierce, Rockford, USA) in a chemilumescence imager (MultiImage Light Cabinet, Alpha Innotech, San Leandro, USA).

Modulation of CD44s shedding by peptides covering putative ADAM10-CD44s-interaction motives
6 x 10^4 cells of *Bro* or *HT144* cells were seeded into a 24 well tissue culture plastic plate. After 10h, cells were washed twice with PBS and the medium was replaced by conditioned medium consisting of the peptides covering the ADAM10 minimal binding sites (Table 1) or DMSO as control. Peptides were used at a final concentration of 7.5 µM in serum free medium. After 24h the shed solCD44s in the supernatant was determined using a CD44 ELISA.

Western Blot
For western blot analysis of solCD44s, cell free supernatant of either *Bro* or transfected CHO cells were collected and concentrated twenty-fold using Vivaspin® 15 Centrifugal Concentrators (Vivaproducts). Equal amounts of the supernatant concentrates were subjected to 10% Tris-Glycin SDS-PAGE under reducing conditions. Proteins were transferred onto PVDF membranes (Hybond-P; GE-Healthcare). Blocking was performed for 1 hour with 5% non-fat dried milk powder in PBS. The membranes were incubated with mouse anti-CD44 monoclonal antibody (SFF304, 1µg/ml in blocking buffer) followed by HRP conjugated secondary antibody and chemiluminescence substrate (ECL, Pierce, Rockford, USA) incubation. Chemiluminescent signals were detected on a luminescent image analyser (INTAS, Münster, Germany).

Acknowledgements
This work was supported by grants of the Deutsche Forschungsgemeinschaft DFG-SFB 610 B7 to JCS.

Reference List

[1] Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003; 4(1):33-45.

[2] Ahrens T, Assmann V, Fieber C, Termeer C, Herrlich P, Hofmann M, Simon JC. CD44 is the principal mediator of hyaluronic-acid-induced melanoma cell proliferation. J Invest Dermatol 2001; 116(1):93-101.

[3] Ahrens T, Sleeman JP, Schempp CM, Howells N, Hofmann M, Ponta H, Herrlich P, Simon JC. Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid. Oncogene 2001; 20(26):3399-3408.

[4] Goebeler M, Kaufmann D, Brocker EB, Klein CE. Migration of highly aggressive melanoma cells on hyaluronic acid is associated with functional changes, increased turnover and shedding of CD44 receptors. J Cell Sci 1996; 109 (Pt 7):1957-1964.

[5] Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 2001; 15(8):968-980.

[6] Naor D, Sionov RV, Ish-Shalom D. CD44: structure, function, and association with the malignant process. Adv Cancer Res 1997; 71:241-319.

[7] Hanagiri T, Shinohara S, Takenaka M, Shigematsu Y, Yasuda M, Shimokawa H, Nagata Y, Nakagawa M, Uramoto H, So T, Tanaka F. Effects of hyaluronic acid and CD44 interaction on the proliferation and invasiveness of malignant pleural mesothelioma. Tumour Biol 2012; 33(6):2135-2141.

[8] Afify A, Purnell P, Nguyen L. Role of CD44s and CD44v6 on human breast cancer cell adhesion, migration, and invasion. Exp Mol Pathol 2009; 86(2):95-100.

[9] Yu Q, Stamenkovic I. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev 1999; 13(1):35-48.

[10] Stamenkovic I, Aruffo A, Amiot M, Seed B. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J 1991; 10(2):343-348.

[11] Misra S, Heldin P, Hascall VC, Karamanos NK, Skandalis SS, Markwald RR, Ghatak S. Hyaluronan-CD44 interactions as potential targets for cancer therapy. FEBS J 2011; 278(9):1429-1443.

[12] Hernandez D, Miquel-Serra L, Docampo MJ, Marco-Ramell A, Bassols A. Role of versican V0/V1 and CD44 in the regulation of human melanoma cell behavior. Int J Mol Med 2011; 27(2):269-275.

[13] Saftig P, Reiss K. The "A Disintegrin And Metalloproteases" ADAM10 and ADAM17: novel drug targets with therapeutic potential? Eur J Cell Biol 2011; 90(6-7):527-535.

[14] van Kilsdonk JW, van Kempen LC, van Muijen GN, Ruiter DJ, Swart GW. Soluble adhesion molecules in human cancers: sources and fates. Eur J Cell

Biol 2010; 89(6):415-427.
[15] Guo YJ, Liu G, Wang X, Jin D, Wu M, Ma J, Sy MS. Potential use of soluble CD44 in serum as indicator of tumor burden and metastasis in patients with gastric or colon cancer. Cancer Res 1994; 54(2):422-426.
[16] Ristamaki R, Joensuu H, Lappalainen K, Teerenhovi L, Jalkanen S. Elevated serum CD44 level is associated with unfavorable outcome in non-Hodgkin's lymphoma. Blood 1997; 90(10):4039-4045.
[17] Nagano O, Murakami D, Hartmann D, De SB, Saftig P, Iwatsubo T, Nakajima M, Shinohara M, Saya H. Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. J Cell Biol 2004; 165(6):893-902.
[18] Anderegg U, Eichenberg T, Parthaune T, Haiduk C, Saalbach A, Milkova L, Ludwig A, Grosche J, Averbeck M, Gebhardt C, Voelcker V, Sleeman JP, Simon JC. ADAM10 is the constitutive functional sheddase of CD44 in human melanoma cells. J Invest Dermatol 2009; 129(6):1471-1482.
[19] Kajita M, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J Cell Biol 2001; 153(5):893-904.
[20] Murai T, Miyazaki Y, Nishinakamura H, Sugahara KN, Miyauchi T, Sako Y, Yanagida T, Miyasaka M. Engagement of CD44 promotes Rac activation and CD44 cleavage during tumor cell migration. J Biol Chem 2004; 279(6):4541-4550.
[21] Nakamura H, Suenaga N, Taniwaki K, Matsuki H, Yonezawa K, Fujii M, Okada Y, Seiki M. Constitutive and induced CD44 shedding by ADAM-like proteases and membrane-type 1 matrix metalloproteinase. Cancer Res 2004; 64(3):876-882.
[22] Okamoto I, Kawano Y, Tsuiki H, Sasaki J, Nakao M, Matsumoto M, Suga M, Ando M, Nakajima M, Saya H. CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. Oncogene 1999; 18(7):1435-1446.
[23] Ueda J, Kajita M, Suenaga N, Fujii K, Seiki M. Sequence-specific silencing of MT1-MMP expression suppresses tumor cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumors. Oncogene 2003; 22(54):8716-8722.
[24] Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. Mol Aspects Med 2008; 29(5):258-289.
[25] Garton KJ, Gough PJ, Raines EW. Emerging roles for ectodomain shedding in the regulation of inflammatory responses. J Leukoc Biol 2006; 79(6):1105-1116.
[26] Mullberg J, Althoff K, Jostock T, Rose-John S. The importance of shedding of membrane proteins for cytokine biology. Eur Cytokine Netw 2000; 11(1):27-38.
[27] Reiss K, Saftig P. The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. Semin Cell Dev Biol 2009; 20(2):126-137.

[28] Guo J, He L, Yuan P, Wang P, Lu Y, Tong F, Wang Y, Yin Y, Tian J, Sun J. ADAM10 overexpression in human non-small cell lung cancer correlates with cell migration and invasion through the activation of the Notch1 signaling pathway. Oncol Rep 2012; 28(5):1709-1718.

[29] Scheller J, Chalaris A, Garbers C, Rose-John S. ADAM17: a molecular switch to control inflammation and tissue regeneration. Trends Immunol 2011; 32(8):380-387.

[30] Weber S, Saftig P. Ectodomain shedding and ADAMs in development. Development 2012; 139(20):3693-3709.

[31] Lee SB, Schramme A, Doberstein K, Dummer R, Abdel-Bakky MS, Keller S, Altevogt P, Oh ST, Reichrath J, Oxmann D, Pfeilschifter J, Mihic-Probst D, Gutwein P. ADAM10 is upregulated in melanoma metastasis compared with primary melanoma. J Invest Dermatol 2010; 130(3):763-773.

[32] Zigrino P, Mauch C, Fox JW, Nischt R. Adam-9 expression and regulation in human skin melanoma and melanoma cell lines. Int J Cancer 2005; 116(6):853-859.

[33] Nagano O, Saya H. Mechanism and biological significance of CD44 cleavage. Cancer Sci 2004; 95(12):930-935.

[34] Caescu CI, Jeschke GR, Turk BE. Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10. Biochem J 2009; 424(1):79-88.

[35] Reddy P, Slack JL, Davis R, Cerretti DP, Kozlosky CJ, Blanton RA, Shows D, Peschon JJ, Black RA. Functional analysis of the domain structure of tumor necrosis factor-alpha converting enzyme. J Biol Chem 2000; 275(19):14608-14614.

[36] Smith KM, Gaultier A, Cousin H, Alfandari D, White JM, DeSimone DW. The cysteine-rich domain regulates ADAM protease function in vivo. J Cell Biol 2002; 159(5):893-902.

[37] White JM. ADAMs: modulators of cell-cell and cell-matrix interactions. Curr Opin Cell Biol 2003; 15(5):598-606.

[38] Janes PW, Saha N, Barton WA, Kolev MV, Wimmer-Kleikamp SH, Nievergall E, Blobel CP, Himanen JP, Lackmann M, Nikolov DB. Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell 2005; 123(2):291-304.

[39] Lockshin A, Giovanella BC, De Ipolyi PD, Williams LJ, Jr., Mendoza JT, Yim SO, Stehlin JS, Jr. Exceptional lethality for nude mice of cells derived from a primary human melanoma. Cancer Res 1985; 45(1):345-350.

[40] van Muijen GN, Danen EH, Veerkamp JH, Ruiter DJ, Lesley J, van den Heuvel LP. Glycoconjugate profile and CD44 expression in human melanoma cell lines with different metastatic capacity. Int J Cancer 1995; 61(2):241-248.

[41] Muller MQ, de Koning LJ, Schmidt A, Ihling C, Syha Y, Rau O, Mechtler K, Schubert-Zsilavecz M, Sinz A. An innovative method to study target protein-drug interactions by mass spectrometry. J Med Chem 2009; 52(9):2875-2879.

[42] Parkin E, Harris B. A disintegrin and metalloproteinase (ADAM)-mediated ectodomain shedding of ADAM10. J Neurochem 2009; 108(6):1464-1479.

[43] Edlich F, Maestre-Martinez M, Jarczowski F, Weiwad M, Moutty MC, Malesevic M, Jahreis G, Fischer G, Lucke C. A novel calmodulin-Ca2+ target recognition activates the Bcl-2 regulator FKBP38. J Biol Chem 2007; 282(50):36496-36504.

[44] Frank R, Dubel S. Analysis of protein interactions with immobilized Peptide arrays synthesized on membrane supports. CSH Protoc 2006; 2006(4).