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ADAM15 decreases RGD-dependent integrin αvβ3-mediated ovarian cancer cell adhesion to vitronectin without affecting cell proliferative activity

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1. INTRODUCTION

1.1 Ovarian Cancer

Ovarian cancer is one of the most frequent and lethal gynecological malignancies in the western hemisphere, second only to endometrial cancer. Mortality rates in Europe range from 3.6/100,000 in Portugal to 9.3/100,000 in Denmark (Bray et al., 2005, 977-90). The ethiology of most ovarian cancers remains unclear so far. The vast majority of cancers are sporadic resulting from the accumulation of genetic damage over a woman's lifetime. Some risk factors, such as age, environmental and nutritional factors, infertility, and nulliparity seem to be of importance in the development of ovarian cancer. Approximately 10% of all ovarian cancers arise in women who have inherited mutations in cancer susceptibility genes BRCA1 and BRCA2 in chromosomes 17 and 13, respectively.

Some factors are known to be protective, such as the number of pregnancies and the duration of the intake of contraceptives. Recent data show that the incidence of the BRCA1/2-associated ovarian cancer can be decreased by the intake of contraceptives, even though this is assumed to increase the risk of early-onset breast cancer (Narod et al., 2002, 1773-9).

The microenvironment of ovarian cancer cells and the phenomenon of local tissue remodeling as the initial step of tumor metastasis have been of great interest in the research of ovarian cancer. Ovarian cancer cell adhesion to and detachment from the **e**xtra**c**ellular **m**atrix (ECM) is, at least partially, mediated by the ECM component vitronectin (VN) as a primary adhesion substrate of **o**varian **s**urface **e**pithelial (OSE) as well as cancer cells. Adhesion to the ECM is mediated by a family of cell adhesion receptors, the integrins, among which integrin $\alpha v\beta 3$ is of special importance. The human ovarian cancer cell line OV-MZ-6 expresses integrin $\alpha v\beta 3$, which interacts with VN. In fact, a large proportion of both normal OSE and highly differentiated ovarian tumors co-express $\alpha v\beta 3$ and VN (Carreiras et al., 1996, 260-7). It has been shown that $\alpha(v)$ integrins promote OSE cell adhesion on VN but not on fibronectin (FN), another ECM component (Cruet et al., 1999, 254-60). Human ovarian adenocarcinoma cells actively synthesize VN to organize their adhesion to the ECM and subsequent metastatic growth via interaction with integrin $\alpha v\beta 3$ (Carreiras et al., 1999, 312-22).

Diagnosis of ovarian cancer often occurs at an advanced stage since the carcinoma shows symptoms late and subtly. Besides, most patients and too many physicians are frequently not aware of the potential seriousness of the rather harmless and unspecific symptoms. New approaches in the attempt to diagnose ovarian cancer at an earlier stage, such as genomic

technologies and proteomics promise to be more sensitive and specific but are not yet commonly used (Alexe et al., 2004, 766-83; Conrads et al., 2004, 163-78; Rapkiewicz et al., 2004, 2604-12).

Therapy of ovarian cancer mostly relies on a combination of radical surgical debulking and chemotherapy with paclitaxel and carboplatin as potent drugs. Remission times are oftentimes followed by recurrences, however, and toxic side effects of the chemotherapy regimens are common. Other approaches, like gene therapy targeted at p53 mutations do not appear to fulfill initial expectations either (Zeimet et al., 2003, 415-22). Outcomes of the conventional combination therapy have been unsatisfying so far, and the search for new methods of early diagnosis and more efficient treatment is ongoing. Hereby, particular emphasis has been placed on early diagnosis, as this seems to be a more promising approach regarding survival rates than extensive treatment.

1.2 Cancer metastasis and the local ECM microenvironment

Tumor metastasis is the leading cause of death in cancer patients, and the steps required for metastasis are similar to all tumors: *proteolysis*, *cell adhesion*/*detachment*, *cell migration*, *angiogenesis*, and *metastatic growth* (Woodhouse et al., 1997, 1529-37). Early tumor metastasis largely depends on the complex interaction between malignant cells and their surrounding microenvironment, in particular the ECM. The remodeling of the ECM which is at first limited to the immediate pericellular environment is considered to be an essential process in local invasion (Liotta et al., 2001, 375-9). A decisive factor in the metastatic process is the balance between the formation and loosening of adhesive cell contacts which is largely based on the regulated expression of integrins (Hapke et. al., 2003, 1073-83).

However, cellular abilities like adhesion to and detachment from, proteolysis of, and migration within the ECM are not restricted to *malignant* cells. The very same steps are taken, e.g. during *benign* processes like angiogenesis, embryogenesis, wound healing, and morphogenesis (Woodhouse et al., 1997, 1529-37).

The interaction between the malignant cell and the ECM plays a crucial role in maintaining the balance between controlled growth or regeneration and uncontrolled, malignant proliferation. This could be demonstrated, e.g. during the cyclic control of the ovary before menopause and led to the assumption that, after the menopause, the loss of this physiological milieu might be involved in the promotion of epithelial ovarian cancer (Liotta et al., 2001, 375-9; Nillson et al., 2001, 479-86). Malignant ovarian cells have escaped physiological control of these physiological remodeling processes, which maintain a balance between proliferation, growth, and apoptosis. An important role in most malignant settings is also attributed to the surrounding stromal cells of the ECM, since these cells synthesize and

secrete most of the enzymes and inhibitors found at the invasive front of the tumor (Liotta et al., 2001, 375-9).

Prior to local invasion of malignant cells, proteolysis of the ECM by, e.g. **m**atrix **m**etallo**p**roteases (MMPs) is required. This has been demonstrated in various tumors, such as ovarian cancer (Curran et al., 1999, 300-8; Garbett et al., 2000, 99-106; McCawley et al., 2000, 149-56; John et al., 2001, 14-23; Kerkela et al., 2002, 258-69). The local ECM facilitates synthesis of the MMPs by largely unknown mechanisms. This means that a system of mutual support between the malignant cell and the ECM is essential for successful metastasis.

Another crucial step in metastasis is cell migration (Palm et al., 2005, 396-404). In order to migrate within the tumor environment, malignant cells have to be capable of both adhering to and detaching from the ECM in a carefully controlled and fine-balanced system (Hapke et al., 2003, 1073-83). The most effective migration is supposed to occur at an intermediate level of adhesion of the migrating malignant cell to the ECM (Herren et al., 2001, 152-60).

In malignant cells, an adapted expression of adhesion molecules, such as cadherins, selectins, and integrins has been demonstrated (Kurschat et al., 2002, 482-9). Expression levels of adhesive molecules have been found to correlate with tumor progression and malignancy in a variety of tumors (Felding-Haberman et al., 2002, 427-36; Trikha et al., 2002, 2824-33, Calvo et al., 2002, 5325-35, Okegawa et al., 2002, 1836-43, Hendrix et al., 2003, 151-61, Hapke et al., 2003, 1073-83).

The relative composition of the ECM is thought to change according to the respective stage of local cell invasion. Cells hereby range from immune cells and inflammatory cells to myofibroblasts and vascular cells. Other highly important components of the ECM are collagens (Col), glycoproteins (laminin [LN], FN, tenascin, and VN), proteoglycanes, and polysaccharides (Liotta et al., 2001, 375-9). Amongst these ECM proteins, VN has evolved into an ECM component of special interest due to its interaction with integrins involved in metastasis and cancer growth, such as integrin $\alpha v\beta 3$.

1.2.1 The role of integrins in benign and malignant cell functions

Cells are controlled by their ECM microenvironment to a great extent. The effects of the distinct ECM proteins are primarily mediated by integrins, a family of cell adhesion receptors. These heterodimeric, cell surface-anchored proteins mediate signals between cells, and between cells and the ECM (Ruoslahti et al., 1999, 1028-32).

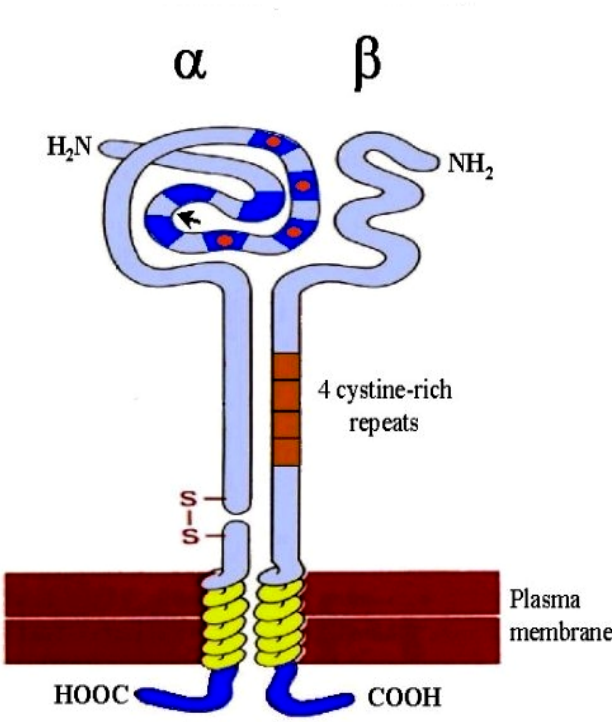


Figure 1 Molecular scheme of an integrin molecule
Integrins are heterodimeric molecules comprised of an α and a β chain and are anchored in the cell membrane. Dark blue regions represent homologous repeats. Red dots denote binding sites for divalent metal ions. The extracellular domain contains a 4 cystein-rich repeat region (orange) in the β chain and a disulfid-bond in the α chain. With their extracellular domain they are capable of binding to various ECM ligands. Interaction of, i.e. integrin αvβ3 with the ECM protein VN occurs via the amino acid triplet RGD (Arg – Gly – Asp). Many integrins possess a proteolytic cleavage site for release into the ECM

Integrins comprise an α and a β subunit, which are non-covalently linked to the cell surface. Both subunits contain a large extracellular domain, a transmembrane domain, and a small cytoplasmic tail. There is a large number of integrins composed of different α and β subunits, which are combined in a multitude of ways. Most integrins interact with a number of different ECM proteins and, conversely, ECM proteins, such as FN, LN, Col, and VN bind to several integrins (Ruoslahti et al., 1999, 1028-32). Interaction of integrins with the ECM is, e.g.in the case of αvβ3 and VN mediated by the recognition motif RGD located within VN while, e.g. both alpha(1)beta(1) and alpha(2)beta(1) integrins have been shown to recognize triple-helical GFOGER (Gly-Phe-Hydroxyproline-Gly-Glu-Arg) found in collagens (Zhang et al., 2003; 7270-7).

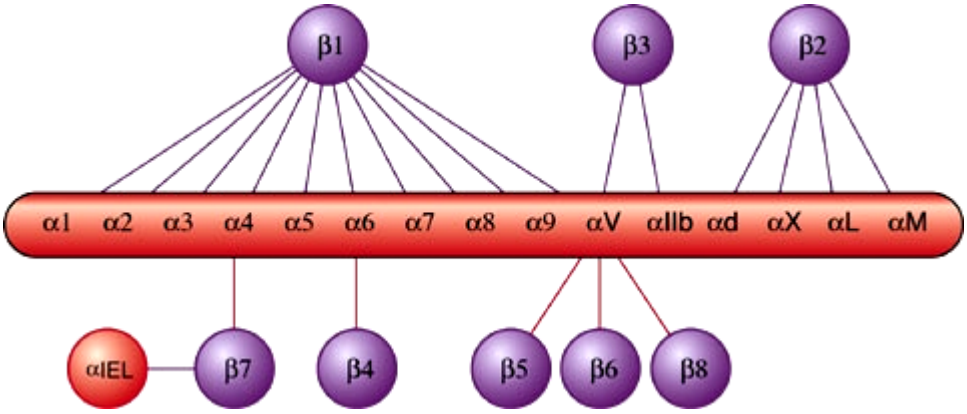


Figure 2 Combinations of different α and β subunits of integrins
Integrin molecules are constituted of a variety of different α and β subunits. These can be combined in a multitude of combinations resulting in a large number of different integrin molecules. The αv-, the β3–, and the β1-subunit are of particular importance in malignant environments.

An important feature of integrins is that they are capable of both “inside-out” signaling and “outside-in” transmission of signals between the cytoplasm and the ECM. This means that they can transmit signals from the ECM into the cell via the intracellular signaling cascade and from the cytoplasm outside into the ECM. Integrins are crucial during various physiological and malignant processes, such as, i.e. cell migration. Hereby, integrins constitute the tip of the filopodia, bind to the ECM, and trigger the initiation of focal adhesions (Ruoslahti et al., 1999, 1028-32). Apart from being mere adhesive receptors, integrins are involved in signal transduction. These pathways have been investigated recently as targets for new drugs. Since cytoplasmic tails of integrins lack enzymatic activity, integrins can function by clustering on the cell surface and subsequent association with intracellular (enzymatic) adapter proteins leading to further downstream signaling within the cellular signaling cascades. The adapter proteins facilitate contact between integrin tails and components of the cytoskeleton, cytoplasmic kinases, and growth factor receptors (Ruoslahti et al., 1999, 1028-1032). This can in turn lead to cell migration, cell growth or, conversly, apoptosis.These processes can be mediated by, e.g. focal adhesion kinases (FAK). These molecules are, e.g. capable of regulating cell migration through controlled adhesion and detachemnt of cells by regulating the formation of a leading (adhesive) edge during cell migration and by coordinating integrin signaling to direct the correct spatial activation of membrane protrusion (Tilghman et al., 2005, 2613-23).

Among the family of integrins, αvβ3 is of particular importance, since it is involved in the proliferation and progression of a number of cancers, such as ovarian cancer (Hapke et al., 2003, 1073-83).

1.2.2 Integrin αvβ3

Integrin αvβ3 is one of the best characterized integrins binding preferably to the amino acid (aa) recognition triplet RGD (Arg-Gly-Asp). This aa sequence was first associated with binding of the RGD-motif of FN to integrin α5β1 (Suehiro et al., 2000, 705-10). The three-dimensional extracellular (EC) domain of integrin αvβ3 has recently been found crystallized in a bent conformation considered to be inactive unless it is fully extended by activating stimuli. Upon activation, the then extended EC domain was found to bind to the ligand, FN, possibly in an RGD-dependent manner. Hereby the $Mn^{(2+)}$-bound EC domain of integrin αvβ3 built a stable, soluble complex with FN (Adair et al., 2005; 1109-18).

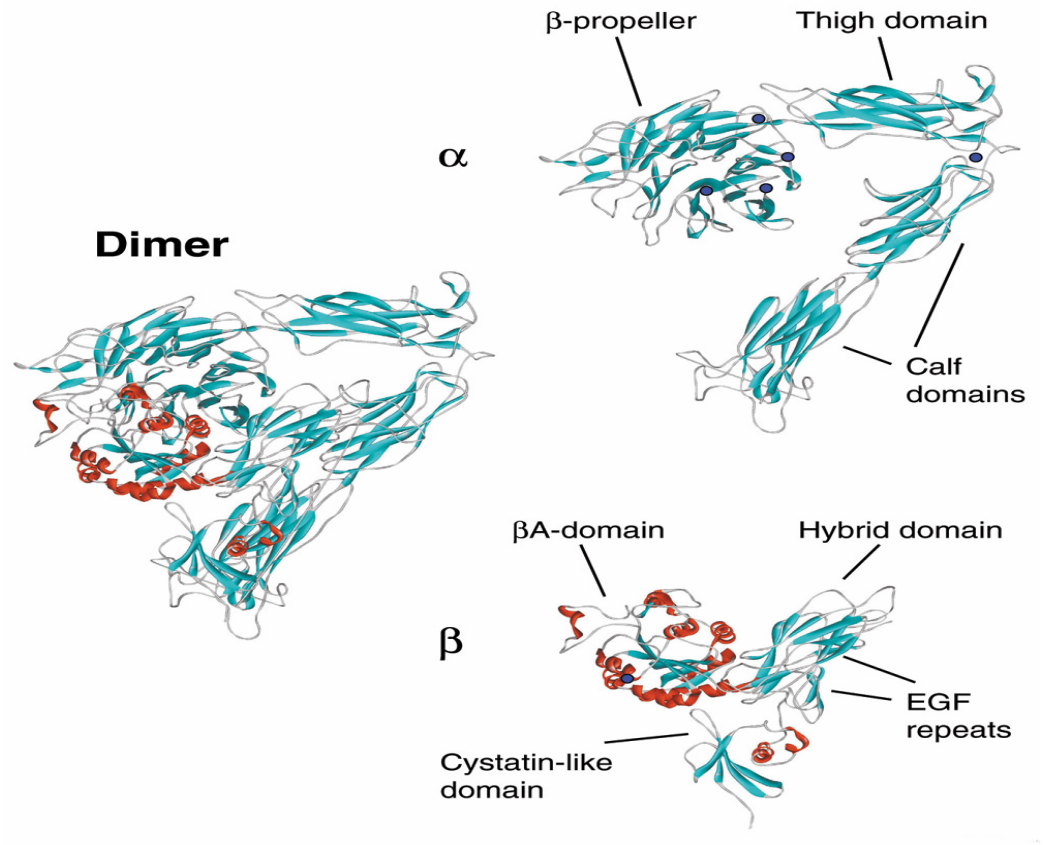


Figure 3 Crystal structure of integrin αvβ3
Crystal structure of integrin αvβ3 showing the dimer and individual subunits. The domains that make up each integrin subunit (α- and β-subunits) are shown. Secondary structure elements are shown as red α-helices or cyan β-strands/ribbons. Blue circles represent the six cation-binding sites. Two of the four epidermal growth factor (EGF) repeats in the β-subunit are not visible in the structure (Humphries et al., 2002, 69-78)

Integrin αvβ3 mainly mediates adhesion to VN and to a number of other ECM proteins, such as FN (Schvartz et al., 1999, 539-44). It is involved in various biological processes, such as cell-ECM interaction (both RGD-dependent and via binding to collagens), cell-cell interaction, bone resorption (highest *in vivo* expression in osteoclasts), and signal transduction (Horton et al., 1997, 721-5).

Elevated expression levels of integrin αvβ3 have been found in tissue sections of invasive ovarian cancer cells when compared to ovarian tumors of low malignant potential (LMP) (Liapis et al., 1997, 443-9). Cannistra and coworkers (1995) found elevated expression levels of integrin αvβ3 both in CAOV-3, SKOV-3, and OVCAR-3 ovarian cancer cell lines as well as in primary ovarian cancer tissue samples. Moreover, OV-MZ-6 ovarian cancer cell line adhesion to VN and proliferation on VN is significantly increased by overexpression of αvβ3 *in vitro* (Hapke et al., 2003, 1073-83).

Integrin αvβ3 has also been investigated as far as tumor cell proliferation and invasiveness are concerned. Hereby, in the ovarian cancer cell lines IGROV1 and SKOV-3, respectively, integrin αvβ3 has been shown to regulate cancer cell proliferation by activating intracellular kinases (Cruet-Hennequart et al., 2003, 1688-702). An enhanced expression of αvβ3 as indicator of increased invasiveness has been found, e.g. in metastasized breast cancer (Gasparini et al., 1998, 2625-34) and malignant melanoma (Albeda et al., 1990, 6757-64; Marshall et al., 1996, 129-38), amongst others.

The interaction between integrin αvβ3 and the RGD motif could be of significant importance, since αvβ3 has also been shown to be involved in functions, such as tumor (neo-)angiogenesis and tumor metastasis (Vacca et al., 2001, 993-1003; Gillan et al., 2002, 5358-64; Beauvais et al., 2003, 219-32; Cruet-Hennequart et al., 2003, 1688-702; Kumar, 2003, 123-31). Indicating its crucial role in (neo-)angiogenesis, integrin αvβ3 has been identified as a *marker* of (neo-)angiogenesis (Gasparini et al., 1998, 2625-34; Kageshita et al., 2000, 314-8; Ellegala et al., 2003, 336-41; De et al., 2005, 7589-94).

Thus, synthetic cyclic RGD peptides and antibodies directed against integrin αvβ3 (Haubner et al., 1999, 1061-71) have been developed and tested both *in vitro* and in clinical trials *in vivo* with promising results in various malignancies, such as breast cancer (Brooks et al., 1995, 1815-22; Harms et al., 2004, 119-28) and prostate cancer (Nemeth et al., 2003, 413-20), amongst others.

Another new anti-tumoral approach is the conjugation of conventional chemotherapeutic agents, such as paclitaxel, with cyclic RGD peptides targeted against integrin αvβ3. This has been examined *in vitro* in the metastatic breast cancer cell line MDA-MB-435. Hereby, the elevated expression of integrin αvβ3 in malignant tumors can be used to selectively

target tumor cells hereby increasing selectivity and reducing toxicity (Chen et al., 2005, 1098-106).
Encouraged by these results the search for naturally occurring integrin αvβ3 antagonists has resulted in the identification of ADAM15.

1.2.3 Vitronectin - a multifunctional component of the ECM

VN is a multifunctional glycoprotein with a molecular weight of 75 kDa, present in blood and in the ECM. It is known to bind to various receptors, such as integrins. Besides, it has been found to bind urokinase-type plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1), heparin, and collagens (Schvartz et al., 1998, 539-44). Considering its location in the ECM and its ability to interact with other ECM components, it is believed to have a role in ECM remodeling eventhough it does not have any protease-like properties itself.

Human ovarian adenocarcinoma cell lines are able to synthesize VN, as revealed by the presence of VN mRNA and the protein *in vitro*, and an enhanced expression of VN has been found, e.g. in ovarian carcinoma cells. Moreover, in ovarian cancer cell lines *in vitro*, VN is organized into a particular pattern in combination with the recruitment of alphav integrins into focal contacts (Carreiras et al., 1999(b), 285-94).
Immunohistochemical analyses of extracts of human breast cancer tissue led to the detection of elevated VN levels in the ECM around clusters of breast cancer cells. VNmRNA levels, however, were indistinguishable in normal and malignant breast tissue leading to the hypothesis that the elevated VN protein levels in malignant breast tissue could result form a leakage form blood vessels where VN is thought to play an important role in association with integrins and the plasminogen activation system (Aaboe et al., 2003, 72-82). This is compatibel with the results from Dufourcq and coworkers who were able to show that VN, in association with integrin αvβ3, is involved in neointima formation after vascular injury *in vitro* (Uhm et al., 1999, 1587-94; et al). VN also semms to be involved in the process of natural cell death as VN was found to protect human glioma cell lines D54 and U251 from drug-induced apoptosis *in vitro*.
VN contains an RGD-motif at aa position 45-47, adjacent to the N-terminal Somatomedine B domain. This sequence is a known integrin ligand to, e.g. integrins αvβ3, αvβ5, αvβ1, αvβ6, αvβ8, and α4β7, respectively.

Figure 4 Scheme of the vitronectin molecule
VN and its binding sites for ligands PAI-1 (pos. aa 1-45, 348-370), uPAR (pos. aa 1-45), plasminogen (pos. aa 332-348), heparin (pos. aa 348-361), integrins (pos. aa 45-47 constitutes the RGD-motif), and the thrombin-antithrombin III complex (TAT), and Col which are located in the N-terminus of the molecule. (pos. aa 47 – 64)

Considering the involvement of integrin αvβ3 in angiogenesis, wound healing, bone resorption, and tumor metastasis, RGD-dependent interaction between VN and integrin αvβ3 has evolved into a potential target for various therapeutical interventions, such as anti-cancer drugs. Hence, e.g. cyclic RGD peptides developed to interfere with this interaction are of considerable interest and and are under current clinical investigation (Sheu et al., 1994, 256-63; Horton et al., 1997, 235-54; Schvartz et al., 1998, 539-44; Kessler et al., 1999, 3033-40; Kok et al., 2002, 3465-76; Goodman et al, 2002, 1045-51; Thumshirn et al., 2003, 2717-25, Shannon et al., 2004, 129-38, Haubner et al., 2004, 1439-55). Based on the knowledge about the therapeutic potential of synthetic cyclic RGD peptides as anti-cancer drugs, naturally occurring integrin αvβ3 antagonists displaying an RGD triplet, such as ADAM15 (***A*** ***D***isintegrin ***A***nd ***M***etalloprotease) have become the focus of increased investigation.

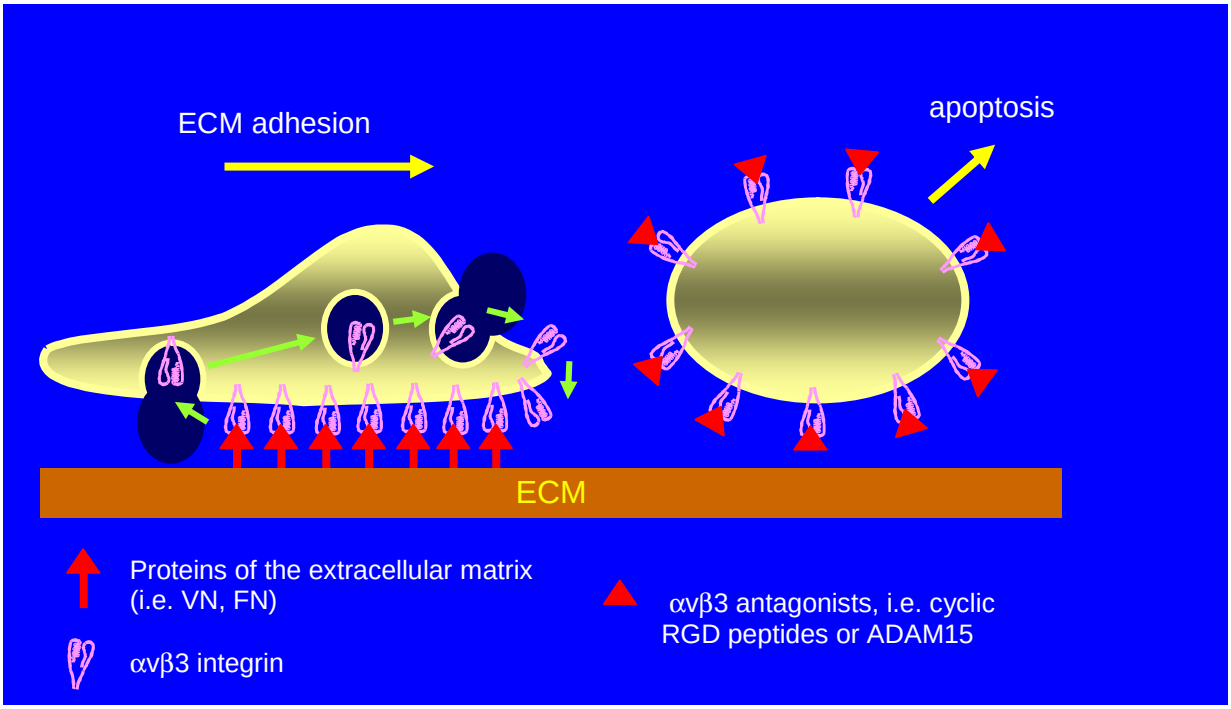


Figure 5 Interruption of integrin αvβ3-mediated binding of ovarian surface epithel and ovarian cancer cells to the ECM by competitive integrin antagonists displaying the amino acid triplet RGD

Many cell lines, such as ovarian cancer cell lines are anchorage-dependent. Disruption of their adhesive capabilities leads to apoptosis. Binding of ovarian surface epithel (OSE) and ovarian cancer cells to the ECM is mediated by, e.g. integrin αvβ3 and ECM proteins like, VN and FN in an RGD-dependent manner. Interruption of this interaction could occur through molecules displaying an RGD-motif and interacting competitively with the cellular integrin αvβ3, such as synthetic cyclic RGD peptides or the naturally occurring integrin antagonist ADAM15 which displays an RGD-motif in its disintegrin domain.

1.3. The ADAM family of proteins

ADAMs are a family of membrane-anchored cell surface proteins, which are part of the group of reprolysins. Reprolysins are a subfamily of the metzincins characterized by the amino acid consensus sequence **H**EXX**H**XXGXX**H** in the catalytic domain. The family of reprolysins also comprises the SVMP (**s**nake **v**enom **m**etallo-**p**roteases), which bear a great homology to the extracellular domains of ADAMs. The SVMPs bind to the platelet integrin αIIb3. This leads to an interruption of the physiological blood coagulation process by preventing platelet aggregation leading to hemorrhage.

Another important subgroup of the metzincin superfamily is the family of matrixins (MMPs).

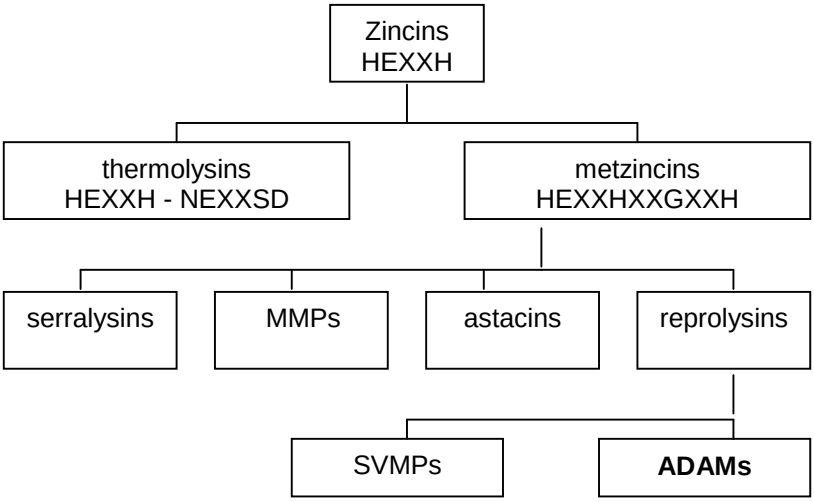


Figure 6 Classification of metalloendopepetidases
HEXXHXXGXXH (single letter code, X is any amino acid residue) constitutes the consensus amino acid motif of zinc-binding metzincin endopeptidases. (MMP = matrix metalloprotease; SVMP = snake venom metalloprotease; ADAM = A Disintegrin And Metalloprotease) (adapted from de Clerck, 2000, 1258-68).

The ADAM proteins, which are also referred to as **MDCs** (**m**etalloprotease **d**isintegrin **c**ysteine-rich) are type I glycoproteins, constituted by several different molecular domains: An N-terminal *signal peptide*, followed by a *prodomain,* a *metalloprotease (MP)* and a *disintegrin* domain, a *cysteine-rich region* containing an *EGF repeat* (**E**pidermal **G**rowth **F**actor), a transmembrane domain, and a cytoplasmic tail (Blobel et al., 1992, 248-52; Wolfsberg et White, 1996, 378-83; Schlöndorff et Blobel, 1999, 3603-17; Blobel, 2002, 83-4).

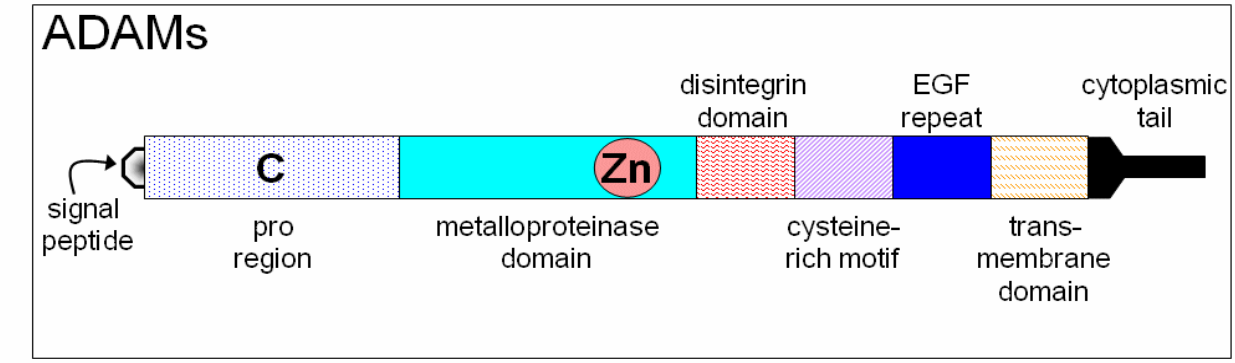


Figure 7 Basic molecular structure of a typical ADAM protein
ADAMs are comprised of a signal peptide (not contained in all ADAMs), a prodomain (C), a metalloprotease domain containing a zinc ion (Zn), a disintegrin domain, a cysteine-rich/EGF domain, a transmembrane domain, and a cytoplasmic tail. Only human ADAM15 contains an additional integrin-binding motif (RGD) in its disintegrin domain.

ADAMs are widely distributed in mammalian organs, such as brain, testis, epididymis, ovary, breast, muscle, placenta, liver, heart, lung, and bone. So far, 34 members of the ADAM family have been discovered.
The main functions ADAMs are involved in are:

- *Proteolysis*
- *Adhesion*
- *Fusion*
- *Protein ecto-domain shedding*
- *Intracellular signaling*

Although all ADAMs share a stably conserved metalloprotease (MP) domain, only 17 ADAMs have been shown to be catalytically active, as demonstrated by their zinc-binding catalytic-site MP consensus sequence (HEXXH). Thus, only a subset of the ADAM family is proteolytically active.
ADAMs were initially described in *fertilization* (Primakoff et al., 1987, 141-49; Blobel et al., 1992, 248-52; Wolfsberg et al., 1993, 10783-7; Almeida et al., 1995, 1095-104), *myoblast fusion* (Yagami-Hirosmasa et al., 1995, 652-56), *protein ecto-domain shedding (angiotensin-converting-enzyme, tumor necrosis factor-α [TNF-α])* (Black et al., 1997, 729-33; Moss et al., 1997, 733-6; Parkin et al., 2004, 423-32), and *neuronal development* (Fambrough et al., 1996, 13233-8).
Böhm (1999) found elevated mRNA and protein levels of ADAM15 in *arthritis* and *chondrosarcoma*. Furthermore, increased levels of ADAM12 have been found in *cardiac hypertrophy* (Asakura et al., 2002, 35-40) and *atrial fibrillation* (Arndt et al., 2001, 620-5). ADAMs have been associated with *gastric Helicobacter Pylori infection* and *carcinoma* (Yoshimura et al., 2002, 332-40), *lung morphogenesis* (Zhao et al., 2001, 623-31), and *Alzheimer´s Disease* (Kojro et al., 2001, 5815-20). Members of the ADAM family have been linked to various physiological and pathophysiological functions. These are, i.e. cell-adhesion to integrins, such as αvβ3 (Zhang et al., 1998, 7345-50; Nath et al., 2000, 2319-28; Cal et al., 2000, 1457-69), α4β1 (Bridges et al., 2003, 3734-41), α9β1 (Eto et al., 2000, 34922-30), and α5β1 (Nath et al., 1999, 579-87).

ADAM	Alternative name	Function and characteristic features	References
1	Fertilin α PH-30 α	Sperm protein, involved in sperm-egg fusion during fertilization	Primakoff et al., 1987; Blobel et al., 1990,1992; Almeida et al., 1995
2	Fertilin β PH-30 β	Interaction with integrin α6β1 on oocytes	Almeida et al., 1995; Cho et al., 1998; Chen et al., 1999a,b,c
3A	Cyristestin1 CYRN 1 tMDC 1	Involved in adhesion and fusion of sperm and oocyte	Frayne et al., 1998; Nishimura et al., 2001
3B	Cyritestin 2 CYRN 2	Both soluble and membrane-bound	Adham et al., 1998
4	tMDC V	Predominantly expressed in testis, function unclear	Wolfsberg et al., 1995
5	tMDC II	Predominantly expressed in testis, function unclear	Frayne et al., 1999
6	tMDC IV	Predominantly expressed in testis	Wolfsberg et al., 1995
7	EPA 1	Involved in sperm maturation	Perry et al., 1995
8	CD 156 MS2	Highly expressed in monocytes; role in (auto)-immune processes; involved in shedding of IgE receptor CD23	Yoshiyama et al., 1997; Fourie et al., 2003
9	MDC 9 Meltrin gamma	Interaction with integrins α5β1 and α6β1; binds to Src homology 3 (SH3) domain of Src; α-secretase activity and cleavage of insulin-B- chain; shedding of heparin-binding (HB)-EGF, high expression in breast and gastric cancer	Howard et al., 1999 ; Koike et al., 1999; Roghani et al., 1999; O'Shea et al., 2003, Lengdeckel et al., 2004, Carl-McGrath et al., 2005
10	Kuzbanian KUZ MADM	α-secretase activity; amyloid precursor protein (APP)-cleavage; involved in axon development in Drosophila melanogaster; processing of NOTCH; cleavage of Col IV, pro-TNF-α; proteolysis of HB-EGF; inhibition by metalloprotease inhibitors (TIMP) 1 + 3	Lunn et al., 1997; Rosendahl et al., 1997; Chubinskaya et al., 1998, Amour et al., 2000; Lemjabbar et al., 2002 ; Kieseier et al, 2003; Hundhausen et al., 2003
11	MDC	Tumor-suppressor gene	Emi et al., 1993
12	Meltrin α	Binding to integrin α9β1 and α-actinin-2; involved in adipogenesis; interaction with SH3 of Src; osteoclast formation; cleavage of α2 macroglobulin; promotes β1-dependent cell spreading; adipogenesis and myogenesis, elevated expression in breast cancer	Eto et al., 2000; Kawaguchi et al., 2002; Thodeti et al., 2003; Kurisaki et al., 2003, Lengdeckel et al., 2004, Roy et al., 2004, Carl-McGrath et al., 2005
13		Interaction of the cytoplasmic domain with intracellular proteins *in vivo*	Alfandari et al., 1997; Cousin et al., 2000
14	ADAM-1 Fertilin-α	Potential function during embryogenic development of *Caenorhabditis elegans*	Pobilewicz, 1996

15	MDC-15 Metargidin	Only human ADAM containing an RGD motif in disintegrin domain; interaction with integrins αvβ3, α5β1, α9β1; type IV collagenase activity; overexpression enhances cell-cell interaction; interaction with SH3-containing endophilin I and SH3PX1; upregulated in rheumatoid, osteoarthritic tissue, and atrial fibrillation; ectodomain cleavage of CD23; increased expression after integrin αvβ3 upregulation; involved in neovascularization, antiangiogenic and antimetastatic activity; *in vitro* and *in vivo,* inhibition of wound healing, aberrant expression in breast cancer, upregulated in gastric cancer	Krätzschmar et al., 1996; Lum et al., 1998; Böhm et al., 1999,2001; Nath et al., 1999; Zhang et al., 1998 Eto et al., 2000; McCulloch et al, 2000; Herren et al., 2001; Poghosyan et al., 2002; Martin et al., 2002; Fourie et al., 2003; Al-Fakhri et al, 2003 ; Horiuchi et al., 2003, Trochon-Joseph et al., 2004, Charrier et al., 2004, Ortiz et al., Carl-McGrath et al., 2005
16	MDC 16	Involved in fertilization of *Xenopus laevis*	Shilling et al., 1997
17	TACE	α-secretase activity; processing of pro TNF-α and TNF-α receptor; inhibition by TIMP-3; activation by nitric oxide; associated with multiple sclerosis; involved in cancer cell motility, elevavated expression in breast cancer	Black et al., 1997; Moss et al., Amour et al., 1998; Peschon et al., 1 Lum et al., 1999; Rio et al., 2000, Zhang et al., 2000; Seifert et al, 2003; Endres et al., 2003; Gschwind et al., 2003, Lengdeckel et al., 2004
18	ADAM 27	Sperm-surface protein	Frayne et al., 1998
19	Meltrin β	Cleavage of α2-macroglobulin; role in osteoblast formation	Inoue et al., 1998; Fritsche et al., 2000; Wei et al., 2001
20, 21		Predominantly expressed in testis	Poindexter et al., 1999:
22,	MDC-2, MD	Predominantly expressed in brain tissue,	Sagane et al., 1998;
23		binding to integrin αvβ3	Cal et al., 2000
24, 25	Testase-1, ·	Testis-specific expression	Zhu et al., 1999
26			
28	MDC-Lm MDC-Ls	Binding to integrin α4β1; only ADAM autocatalytically processing prodomain	Roberts et al., 1999; Howard et al., 2001; Bridges et al., 2001
29,30		Testis-specific expression	Cerretti et al., 1999a,b
31		Preferential expression in Leydig cells	Liu et al., 2000
33		Expressed in mouse tissues, e.g. brain; similar to Xenopus laevi ADAM 13;	Gunn et al., 2002

Table 1 Overview of members of the ADAM family of proteins

1.3.1 Molecular domain structure of ADAMs

The prodomain

The prodomain of all ADAM family members generally comprises about 200 amino acids. Its main function is the inhibition of uncontrolled activity of the MP domain, which is activated only after processing and removal of the prodomain. ADAMs that contain a catalytically

active consensus sequence (HEXXH) indicating proteolytic activity also contain an odd number of cysteine residues in their prodomains. This is thought to be part of a cysteine switch mechanism (Van Wart et al., 1990, 5578-82). Hereby, the zinc (Zn) is coordinated by three histidine residues, with the free sulfhydryl group constituting a fourth coordination site. The MP domain remains inactive unless the prodomain is removed (Schlöndorff et al., 1999, 3603-17). For several ADAMs, like ADAM9, 12, and **15**, furin or furin-like proteases have been demonstrated to cleave the prodomain at a specific recognition sequence (Loechel et al., 1998, 16993-7; Roghani et al., 1999, 3531-40).

The metalloprotease domain

All ADAMs encode a metalloprotease-like domain, which is similar in sequence to Zn-dependent MPs and made up of about 200 amino acids. This domain is present in most ADAMs except for ADAM1 and 2 (Fertilin-α and -β) (Blobel et al., 1992, 248-52). ADAMs whose MP domains contain the amino acid consensus sequence, **H**EXG**H**XXGXX**H**D, are proteolytically active. Structural analysis has shown that the three histidine residues within the MP domain bind a Zn molecule whereas the glutamic acid residue is in charge of the catalytic function of the MP domain (Wolfsberg et al., 1995, 378-83).
Those ADAMs found to contain the Zn-binding HEXXH sequence are ADAM1, 8-10, 12, 13, **15**-17, 19-21, 24-26, 28, 30, and 31, respectively. The substrates both in *vitro* and in *vivo* of many ADAMs still remain unclear. ADAMs not containing the consensus sequence are, e.g. ADAM2-7, 11, 14, 18, 22, 23, and 29, respectively. These ADAMs are catalytically inactive. They may still be active in other functions attributed to ADAMs and mediated by other domains like, e.g. cell adhesion, cell-cell fusion, cell-cell interaction, intracellular signaling, and protein ecto-domain shedding.

The cysteine-rich and EGF domain

The cysteine-rich domain, which contains approximately 160 amino acids, is characterized by the presence of about 10-14 cysteine residues. The main function of this domain is rather unclear so far. Several functions have been implicated by now, such as presentation of the disintegrin domain or involvement in protein-protein interactions (Jia et al., 1996, 1269-76). Some cysteine-rich domains contain a putative fusion peptide (e.g., ASRPVIGTNAVSIETNIPLQQGGRIL peptide sequence in ADAM12-S and 12-L) and could promote membrane fusion (Blobel et al., 1992, 248-52). ADAMs that have been linked to cell-cell fusion are ADAM1, 3, 9, 12-S, and 12-L, respectively (Blobel et al., 1992, 248-52; Wolfsberg et al., 1998, 1310-13). The EGF domain contains about 40 amino acids with 6 cysteine residues and is implicated in interaction with other proteins and in the directing of

the ADAM molecule to its location of functioning. The functions of ADAM12 and 17 give support to this hypothesis:

- The cysteine-rich domain of **ADAM17** is involved in the shedding of the interleukin-1 type-II receptor (Milla et al., 1999, 30563-70)
- The soluble form of **ADAM12** (ADAM12-s) binds to insulin-like growth factor binding protein-3 (IGFBP-3) (Shi et al., 2000, 18574-80). In alliance with the disintegrin domain, the cysteine-rich domain of ADAM 12 is involved in cell-cell adhesion, and the cysteine-rich domain alone was found to adhere to cell-surface proteoglycans (Cao et Zolkiewska, 2001, 24466-72; Cao et al., 2002, 26403-11).

The transmembrane and cytoplasmic domain

Most ADAMs are membrane-anchored type-1 glycoproteins that are integrated into the cell membrane with their hydrophobic sequence. Some ADAMs, however, like the soluble splicing variants of ADAM11, 12, 17, and 28, respectively, lack the transmembrane (TM) and cytoplasmic (CP) domains (Gilpin et al., 1998, 157-66).

CP domains of ADAMs have been implicated in both “inside-out” regulation of activity and “outside in”-signaling (Howard et al., 1999, 31693-9). This means that CP domains can be involved in transmitting signals from stromal cells or the ECM into the cell which leads to association of cytosolic proteins and changes in the cytoskeleton (“outside in”-signaling). Conversely, these events are thought to trigger signals from the cytoplasm via the cell membrane and membrane-anchored integrins to the ECM (“inside-out”-regulation).

The CP domain contains between 40-250 amino acids, and several ADAMs (ADAM1, 7-10, 12, **15,** and 17) have cytosolic signaling motifs. These are, e.g. proline-rich regions resembling Src homology 3 (SH3) ligand domains, which are known to be involved in intracellular signaling events. Based on a multitude of different amino acid sequences and relative positions of these motifs within the ADAM molecule, ADAMs are capable of interacting with a series of different ligands (Poghosyan et al., 2002, 4999-5007).

Given the fact that potential intracellular ADAM functions, such as trafficking to and from the cell surface are highly complex, it is likely that the CP domains are involved in regulating these functions and directing ADAMs to specific cellular compartments (Howard et al., 1999, 31693-9; Poghosyan et al., 2002, 4999-5007). Several ADAMs have been specifically linked to particular intracellular pathways and protein-protein interactions.

- **ADAM9** and **15** have been shown to interact with SH3 domain-containing protein endophilin (Howard et al., 1999, 31693-9). Endophilin is a cytosolic protein involved in the formation of intracellular endocytosis by converting lysophosphatidic acid into arachidonate acid. It complexes with cell surface receptors and signaling molecules.

- **ADAM12** has been demonstrated to interact with the SH3 domains of Src, which is known to initiate cellular signaling events. Src has been found elevated in cancer development and influences apoptosis, cell growth, and adhesion (Frame, 2002, 114-30).

The disintegrin domain

The disintegrin domain of ADAMs contains about 60-90 amino acids and shows a high homology to the disintegrin domain of SVMPs. This domain is supposed to function as a ligand for integrins like, e.g. $\alpha 4\beta 1$ (Bridges et al., 2002, 3784-92), $\alpha 5\beta 1$ (Nath et al., 1999, 579-87), $\alpha 6\beta 1$, $\alpha 9\beta 1$ (Eto et al., 2000, 34922-30), $\alpha v\beta 3$ (Zhang et al., 1998, 7345-50; Nath et al., 1999, 579-87; Cal et al., 2000, 1457-69), and other receptors, such as α-actinin-2 (Galliano et al., 2000, 13933-9).
Usually, disintegrin domains of proteins interact with receptors via a disintegrin loop, e.g. ADAM1 and 2. All ADAMs share certain disintegrin-loop residues, in most cases cysteines (Wolfsberg et al., 1995, 378-83).
All ADAM disintegrin domains except for the one in human (h) ADAM15 lack an RGD motif. Possibly, the disintegrin domain of ADAMs is involved in cell-cell and cell-matrix interactions (Wolfsberg et al., 1995, 275-8; Schlondorff et al., 1999, 3603-17). Eto and coworkers (2000) showed that cell-cell interaction is mediated by interaction of the disintegrin domains of ADAM12 or ADAM15 with integrin $\alpha 9\beta 1$.
SVMPs are soluble disintegrin molecules inhibiting platelet aggregation by binding to integrin $\alpha IIb3$ resulting in hemorrhage. ADAMs may promote rather than disrupt cell-cell interaction since they are mostly membrane-bound molecules (Herren et al., 2001, 152-60).

Several *in vitro* studies so far have identified integrin-binding sites within the disintegrin domains of a few ADAMs:

- The interaction of the disintegrin domain of **ADAM2** (fertilin-β) with integrin $\alpha 6\beta 1$ on oocytes is an important part of fertilization. It is mainly mediated by the tripeptide sequence in the disintegrin domain with a crucial involvement of the amino acid aspartic acid (Chen et al., 1999a, 1-10; Chen et al., 1999b, 549-61; Zhu et al., 2000, 7677-83).
- In studies using the recombinant disintegrin domain of **ADAM3**, sperm-egg binding and fusion could be inhibited. (Takahashi et al., 2001, 809-20).
- A murine **ADAM9** gamma-Fc fusion protein binds to integrin $\alpha 6\beta 1$ on the surface of two fibroblast cell lines. Furthermore, **ADAM9** demonstrated an RGD-independent interaction with integrin $\alpha v\beta 5$ on myeloma cells (Zhou et al., 2001, 574-80).

- **ADAM12** and **15** have both been shown to interact with integrin α9β1 in an RGD-independent manner, thus enhancing cell-cell interaction (Eto et al., 2000, 34922-30).
- **ADAM15** has been demonstrated to interact specifically with integrins αvβ3 and α5β1 in an RGD-dependent way (Krätzschmar et al., 1996, 4593-6; Zhang et al., 1998, 7345-50; Nath et al., 1999, 579-87)
- **ADAM23** has been shown to interact with integrin αvβ3 in an RGD-independent manner (Cal et al., 2000, 1457-69).
- A recombinant Fc fusion protein encompassing the disintegrin domain of **ADAM28** supported adhesion of the T-lymphoma cell line Jurkat in an integrin α4β1-dependent manner (Bridges et al., 2002, 3784-92)

1.4. Human ADAM15

Human ADAM15 is a unique member of the ADAM family of proteins, for it is the only metalloprotease-disintegrin to contain the integrin-binding motif RGD in its disintegrin domain (Krätzschmar et al., 1996, 4593-6).

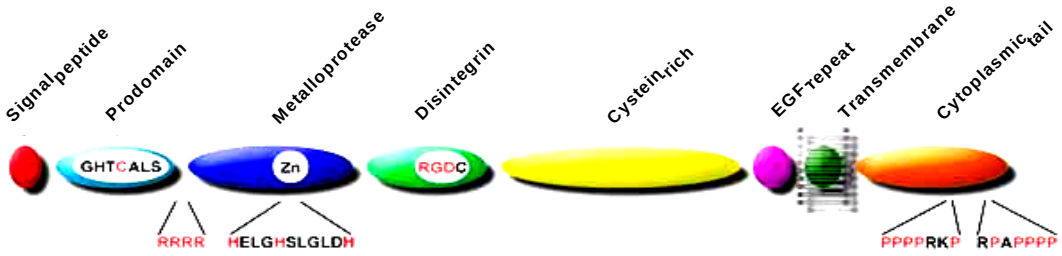


Figure 8 Molecular domains of ADAM15
Represented is the molecular structure of ADAM15. The signal peptide is followed by the prodomain, the metalloprotease domain containing a Zn ion, the disintegrin domain containing the amino peptide triplet RGD, followed by a cysteine rich domain, an EGF-repeat domain, a transmembrane domain, and the cytoplasmic tail.

ADAM15 is also named metargidin indicating that it is part of the family of **META**lloproteases containing an **RGD**-motif in their disintegr**in** domain (**METARGiDIN)**. The mouse and rat

homologues do not contain the RGD motif but instead the sequence TDD (Lum et al., 1998, 26236-47; Bosse et al., 2000, 313-27).
ADAM15 was first cloned by Krätzschmar and coworkers (1996) from MDA-MB-468 mammary epithelial carcinoma cells but was later found to be ubiquitously expressed. A specific co-immunoprecipitation with another 56-kDa protein was shown suggesting that it is part of a protein complex (Krätzschmar et al., 1996, 4593-6).
Chromosomal analysis resulted in assignment of the ADAM15 gene to chromosome 8p22-p11.2, close to loci that have been identified in prostate cancer, accelerated macular degeneration, and Jacob's syndrome (Seldin et al., 2000, 185-7).

1.4.1 Particular features of the molecular domains of ADAM15

ADAM15 shares the classical characteristics and distribution of its molecular domains with other ADAM family members but has some distinct characteristics.

Pro- and metalloprotease domain

Similar to other ADAMs with a Zn-binding consensus sequence within the putative catalytic domain suggesting MP activity, ADAM15 contains an odd number of cysteines in its prodomain. This is thought to regulate the function of the MP domain by a cysteine switch mechanism (Krätzschmar et al., 1996, 4593-6). Unlike most other ADAMs, however, ADAM15 has a modified cysteine switch motif, GHT***C***HLS hinting at other possible ways of activation of the MP domain. Between the prodomain and the MP domain, four consecutive arginine residues form a potential cleavage site for serine proteases like, e.g. the proprotein convertase furin. Moreover, ADAM15 could function as an MP in the intracellular secretory pathway (Lum et Blobel, 1998, 26236-47).
Catalytic activity of the MP domain of ADAM15 has not been shown *in vivo* so far. ADAM15 extracted from mesangial cell membranes was demonstrated to degrade both Col type IV and gelatin *in vitro*, however. Additionally, ADAM15 is increased during migration of human mesangial cells *in vitro*. Migration was inhibited by monoclonal antibodies directed to both the disintegrin and the MP domain. The inhibition of migration after adding a metalloprotease inhibitor suggests a role of the metalloprotease domain in mesangial cell migration. This suggests a potential role for ADAM15 in ECM remodeling through the activity of both the metalloprotease domain and the disintegrin domain (Martin et al., 2002, 33683-9).
A soluble recombinant form of ADAM15 has been shown to catalyze ectodomain shedding of CD23, the low affinity IgE receptor (Fourie et al., 2003, 483-97).

The cysteine-rich domain of ADAM15

The cysteine-rich domain of ADAM15 has not been linked to any specific biological function yet. Since it lacks the typical hydrophobic regions of other potential fusion proteins, an involvement in fusion events seems to be rather unlikely (Krätzschmar et al., 1996, 4593-6; Waters et al., 1997, 1245-54).

The cytoplasmic domain of ADAM15 and its intracellular processing

The CP domain of ADAM15 contains two putative proline-rich SH3 ligand domains. This suggests that interactions with intracellular proteins can affect MP-disintegrin function. Conversely, ADAM15 might function as a mediator in the pathway between the ECM, integrins, and cytoplasm by transmitting signals from the ECM to the cytoplasm (Krätzschmar et al., 1996, 4593-6; Howard et al., 1999, 31693-9).

The CP domain of ADAM15 has been found to interact with endophilin, a protein containing a SH3 domain. Endophilin is a cytosolic protein involved in signal transduction via endocytosis (Howard et al., 1999, 31693-9). ADAM15 has also been identified to interact with SH3PX1 (sorting nexin protein 9), which is a member of a growing family of proteins known as sorting nexins (Howard et al., 1999, 31693-9). They have been linked to the degradation of EGF and vesicular and cell surface receptor trafficking (Worby et al., 2001, 41782-9; Lin et al., 2002, 10134-8). Considering the functions of SH3PX1 and endophilin in intracellular protein trafficking, these proteins could have a function in regulating the subcellular localization or function of ADAM15 (Howard et al., 1999, 31693-9). ADAM15 could function as an intracellular adaptor protein due to its interaction with the SH3 domain of SH3PX1 leaving other intracellular sequences of ADAM15 available for interaction with other proteins.

Intracellular processing of mouse ADAM15 by a furin-type convertase indicates its processing in the trans-Golgi network (Lum et al., 1998, 26236-47).

More recently, the cytoplasmic domain of ADAM15 has been demonstrated to interact specifically with Src family **p**rotein **t**yrosine **k**inase (PTK) members in a phosphorylation-dependent way in hematopoetic cells. Some of the PTKs have been found overexpressed in lymphomas and malignant myeloid cells indicating a potential involvement of ADAM15 in these settings (Poghosyan et al., 2002, 4999-5007). Considering the interactions between ADAM15 and integrins, such as $\alpha v\beta 3$ and $\alpha 5\beta 1$, its main function might be in adhesion that may be subject to control by the Src family PTKs. Conversely, ADAM-mediated adhesion might be capable of regulating PTK function (Poghosyan et al., 2002, 4999-5007).

The disintegrin domain of ADAM15

Containing an RGD-motif, the disintegrin loop of h-ADAM15 stands out amongst all other ADAMs known so far. In fact, the RGD sequence is an important interaction site for integrins, such as αvβ3 or α5β1. The importance of these integrins in pathophysiological settings, such as cancer, angiogenesis, and atherosclerosis has received a substantial degree of attention recently (Varner, 1996, 69-87; Kumar et al., 2000, 169-80; Cooper et al., 2002, 191-4; Kumar, 2003, 123-31; Al-Fakhri et al., 2003, 808-23). Consequently, synthetic RGD peptides have been developed aimed at interfering with integrin-mediated processes (Sulyok, et al., 2001, 1938-50; Thumshirn et al., 2002, 1045-51).

Zhang (1998) first showed RGD-dependent interaction of human ADAM15 with integrin αvβ3. Within this experimental approach, the recombinant ADAM15 disintegrin domain was used as a bacterial fusion protein with glutathione S-transferase in *Ecoli.* Specific binding to integrin αvβ3 in an RGD-dependent manner could be demonstrated. The receptor binding specificity is determined by the flanking sequences, since mutation of RPT**RGD** to NWK**RGD** resulted in binding both to integrin αvβ3 and αIIβ3, the platelet integrin. Herren et al. (1997) demonstrated an increased expression of ADAM15 in endothelial cells suggesting a role of ADAM15 in

(neo-) angiogenesis by binding of ist disintegrin domain to integrin αvβ3 on endothelial cells.

RGD-dependent interaction of ADAM15 with integrins αvβ3 and α5β1, respectively, was demonstrated on haematopoetic cells, the monocytoid cell line U937, and the T-cell line Molt-4 by using RGD-based peptides as inhibitors. Binding of ADAM15 to integrin α5β1 seems to be cell-specific as well as integrin-specific, as α5β1 interaction occured only on Molt-4 cells and not on the monocytoid U937 cell line (Nath et al., 1999, 579-87).

Furthermore, an RGD-independent binding of the recombinant disintegrin domains of ADAM12 and 15 has been shown using mouse ADAM15, which lacks the RGD-motif,

h-ADAM15 mutants lacking the RGD-motif and h- and mouse ADAM12 (Eto et al., 2000, 34922-30). The upregulation of ADAM15 in human osteoarthritic and neoplastic cartilage (Bohm et al., 1999, 1946-51; Bohm et al., 2001, 2046-54), in atherosclerotic settings (Herren et al., 1997, 173-80, Al-Fakhri et al., 2003, 808-23), in intestinal wound healing processes (Charrier et al., 2005, 346-53), and its high expression in monocytes suggests an important role of ADAM15 and its disintegrin domain in the recruitment of cells to inflammatory and malignant sites, and subsequent tissue remodeling (Eto et al., 2000, 34922-30).

1.5. ADAM15 - a potential player in pathophysiological settings

Apart from its associations with various biological processes, such as integrin-binding or intracellular signaling, ADAM15 has been linked to numerous pathophysiological processes.

- Herren and coworkers (1997) were first to describe an upregulation of ADAM15 in atherosclerotic lesions compared to physiological tissue *in vivo* (Herren et al., 1997, 173-80). Upregulation of ADAM15 correlates with the high expression of integrin αvβ3 in endothelial cells and competitive binding of ADAM15 to integrin αvβ3 through its RGD sequence. In fact, an increased expression of ADAM15 following upregulation of integrins αvβ3 and α5β1 was detected in atherosclerosis by Al-Fakhri and coworkers (2003).

  On one hand, ADAM15 could also be involved in the cleavage of adhesive molecules like vascular cell or intracellular adhesion molecule (VCAM-1 and ICAM-1). Both of these adhesion molecules are involved in the recruitment of leukocytes to sites of vascular injury leading to an inflammatory vascular reaction, which is always associated with atherosclerosis. On the other hand, ADAM15 could mediate shedding of anti-adhesive molecules like l-selectin, a leukocyte-specific adhesion molecule. The soluble l-selectin leucocyte adhesion molecules could function as soluble antagonists to an inflammatory response initiated by leukocytes (Herren, 2002, 73-6).

- Horiuchi et al. (2003) showed that mice carrying a targeted deletion of ADAM15 (adam15 (-/-) mice) did not show any phenotypal pathology. They found high levels of ADAM15 mRNA in endothelial cells of normal mice. Adam15(-/-) mice showed a markedly reduced level of (neo-)angiogenesis compared to the wild-type (WT) mice hinting at a role for ADAM15 in (neo-)angiogenesis. Besides, they also found the size of tumors resulting from implanted B16F0 mouse melanoma cells to be significantly smaller in adam15(-/-) mice than in WT controls (Horiuchi et al., 2003, 5614-24).

  In vitro experiments with cultured human aortic smooth muscle cells (SMC) and human umbilical vein endothelial cells (HUVEC) resulted in detection of ADAM15 in both cells, with higher expression levels in SMC. This expression was not mediated by platelet derived growth factor (PDGF) or insulin-like growth factor-I (IGF-I). ADAM15 mRNA levels were not regulated by cell-density, addition of TNF-α or thrombin in HUVEC, either (Herren et al., 1997, 173-80).

- Böhm et al. (1999) discovered an important role of ADAM15 in tissues undergoing extensive matrix remodeling. ADAM15 mRNA is strongly upregulated in osteoarthritic,

rheumatoid and neoplastic tissue (Böhm et al., 1999, 1946-51). Interestingly, the upregulation of ADAM15 mRNA was stronger in mildly damaged tissue indicating that ADAM15 is an important factor in early stage ECM remodeling, e.g. through interaction with integrins, such as $\alpha5\beta1$. Impaired interaction of integrin $\alpha5\beta1$ and ECM has been discovered in malignant cells (Plantefaber et al., 1989, 281-90; Böhm et al., 1999, 1946-51). On the protein level, Böhm et al. (2001) found an enhanced expression of ADAM15 in osteoarthritic and rheumatoid tissue. Considering that the cells that are predominantly involved in early-stage cartilage destruction are $CD68^+$ macrophage-like and $CD68^-$ fibroblast-like synoviocytes, this could be a link to the inflammatory onset (Böhm et al., 2001, 2046-57).

- An increased level of ADAM15 was also found in fibrillating human atria. Hereby, the amounts of ADAM15 mRNA were significantly enhanced compared to sinus rhythm (Arndt et al., 2002, 720-5). The main proportion of ADAM15 was detected in the membrane fraction, whereas, during sinus rhythm the majority was located in the cytoplasmic fraction. Atrial fibrillation is associated with progressive structural changes of the atria, which is in accordance with previous reports on the possibility of ADAM15 involvement in ECM remodeling. Furthermore, the ADAM15/integrin-$\beta1$ ratio is significantly enhanced in atrial fibrillation tissue, indicating a potential role of ADAM15 in integrin shedding. No cleavage products could be detected by immunoprecipitation, however. Consequently, it is possible that ADAM15 interferes with physiological cell-matrix interaction leading to increased cell motility (Arndt et al., 2002, 720-5), cell-cell interaction (Herren et al., 2001, 152-160), and, eventually, atrial dilatation.
- Antiangiogenic and antimetastatic effects of the recombinant h-ADAM15 disintegrin domain (RDD) were demonstrated by Trochon-Joseph and coworkers (2004) both *in vitro* and *in vivo*. RDD showed inhibitory effects on endothelial cell proliferation and adhesion to the ECM proteins FN and VN *in vitro*. Most capillary formation was blocked by RDD in a fibrin gel *in vitro* assay. *In vivo* assays with human mammary adenocarcinoma cells showed a 78% reduction of tumor growth and tumor angiogenesis. The rate of B16F10 melanoma lung metastases in C57BL/6 mice was reduced by 74% *in vivo*.
- An involvement of ADAM15 in breast cancer settings *in vitro* was investigated by Ortiz and coworkers (2004) and Lengdeckel and coworkers (2004). Lengdeckel was unable to demonstrate elevated levels of ADAM15 mRNA in breast cancer specimens. Ortiz was able to show that - while conventional ADAM15 mRNA levels were not increased - an aberrant alternative exon use and an increased copy number of the ADAM15 gene could

be found in breast cancer cells. Hereby, the erratic use of three alternative ADAM15 exons leads to aberrant combinations of ADAM15 mRNA. He concludes that alternative exon usage may be a useful target for breast cancer diagnostics as these could be examined via polymerase chain reaction (PCR)

- The expression of ADAM15 and integrin αvβ3 in lung carcinoma tissue and cell lines was investigated by Schutz and coworkers (2005). Normal and malignant lung tissue were stained with antibodies to the ectosolic and cytosolic domain of ADAM15 and αvβ3 integrin complex. The results were scored (0-12, according to Remmele's score). Normal epithelial cells of the lung were negative or slightly positive for ADAM15 (score<2). The score was always significantly higher for tumor cells. ADAM15 and integrin αvβ3 were often found to be co-localized in lung carcinoma tissue.

2. MATERIAL AND METHODS

2.1 Cell lines

- **OV-MZ-6**
  The human ovarian cancer cell line OV-MZ-6 was isolated and established from the ascites of an ovarian serous-papillary cystadenocarcinoma (Möbus et al., 1992).

- **CHO** (**C**hinese **H**amster **O**vary)
  The ovarian cell line CHO is an established cell line commonly used for the expression of recombinant proteins and was purchased from ATTC, Rockville, MD, USA (Puck, 1958).

Establishment of eukaryotic cell lines overexpressing ADAM15 and variants thereof

The generation of these cell lines was not part of the present dissertation and was kindly provided by Dr. Harald Geppert, Dept. of Chemistry, University of Bielefeld.

Cell transfections and stable (over)expression of ADAM15 and its variants

Transfection of OV-MZ-6 cells with the different ADAM15 cDNA variants and isolation of transfected OV-MZ-6 cell clones were conducted upon neomycin selection as described earlier (Hapke et al., 2001, 26340-8). Several OV-MZ-6 cell clones isolated from each transfection category were tested for ADAM15 expression by immunostaining and subsequent evaluation by confocal laser scanning microscopy (CLSM) as shown below. For the generation of the respective human ADAM15 expressing CHO cell clones, the same procedures were performed as outlined for OV-MZ-6 cells.

In order to characterize the ecto-domain (EC) of ADAM15, cDNA encoding for this domain was amplified using primers Sig-Hind/F-EGFF-Xba-R. Then it was ligated into the vector pcDNA3.1 Myc-His A (Invitrogen, Leek, The Netherlands) via the restriction sites *Hind III* and *Xba I*. DNA encoding for the full-length (FL) ADAM15 molecule was ligated analogously into the vector pcDNA3.1 Myc-His A via the restriction sites *Hind III* and *Xba I* using primers Sig-Hind-F/ZPF-Xba-R. Fusion with a C-terminal c-Myc-epitope-Hexa-Histidine-*tag* was to ensure detection and characterization of the heterologously expressed proteins with an anti-Myc-antibody.

The specific primers used for the cloning into the vector pcDNA 3.1 Myc-His A were:

Sig-Hind-F 5´-AAA AAA AGC TTA TGC GGC TGG CGC TGC TC-3´
EGFF-Xba-R 5´-AAA AAT CTA GAG CTG GTT GCT TTG AGC TGA-3´
ZPF-Xba-R 5´-AAA CAT CTA GAG AGG TAG AGC GAG GAC AC-3´
SGA-Mut 5´-GTG TCG TCC TAC CAG TGG GGC TTG TGA CTT GCC TG-3´

In addition, ADAM15 mutants were generated in which within the disintegrin domain, Arg^{484} was changed to Ser and Asp^{486} to Ala, resulting in the non-integrin binding motif SGA. For this in vitro site-directed mutagenesis, the primer 5' –GTG TCG TCC TAC CAG TGG GGC TTG TGA CTT GCC TG-3' and the kit Transformer™ (Clontech) was used.

OV-MZ-6 and CHO cells were transfected with the following constructs:

- cDNA encoding full-length (FL) ADAM15 containing the RGD motif in the disintegrin domain, in the following referred to as **A-15 FL-RGD**
- cDNA encoding full-length ADAM15 containing an RGD-mutation in the disintegrin domain, in the following referred to as **A-15 FL-SGA**
- cDNA encoding the extracellular (ecto) domain of ADAM15 lacking the transmembrane and cytoplasmic domain and containing the RGD motif in the disintegrin domain, in the following referred to as **A-15 EC-RGD**
- cDNA encoding the ecto domain of ADAM15 lacking the transmembrane and cytoplasmic domain and containing and RGD-mutation in the disintegrin domain, in the following referred to as **A-15 EC-SGA**

Protein expression was verified by immunostaining using antibodies directed against the c-myc epitope and evaluated by CLSM as shown in the following (Fig. 9,10,11). In addition, immunostainings were performed using an antibody directed to the extracellular domain of ADAM15, provided by C. Blobel, Memorial Sloan-Kettering Cancer Center, New York.

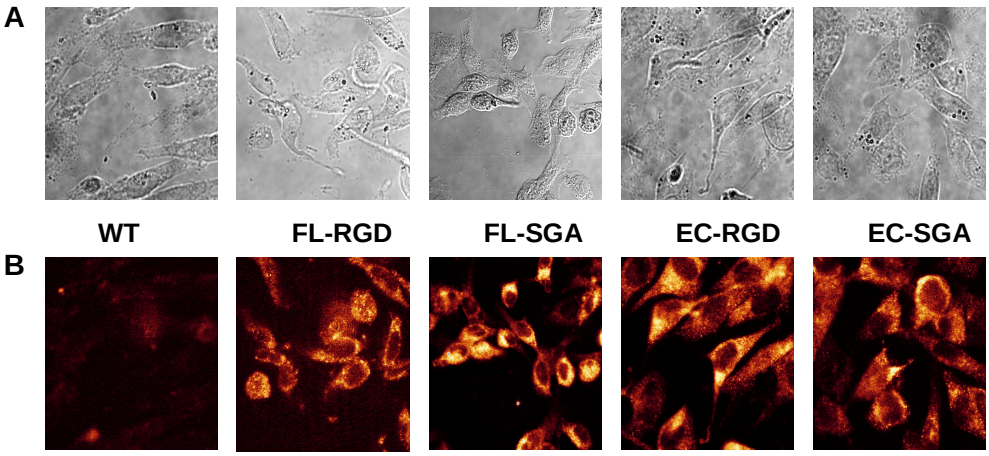


Figure 9 Immunostaining (CLSM) of representative stably transfected isolated ADAM15 CHO cells.
A: Transmission; **B**: Fluorescence**;** (pictures kindly provided by PD Dr. U. Reuning)
Primary antibody: anti-Myc (1 ug/ml)
Secondary antibody: anti-mouse IgG-Alexa-488 (1 ug/ml).

Since CHO WT cells are of hamster origin, they do not endogenously express h-ADAM15. ADAM15 transfected CHO cells show a marked expression of ADAM15, intracellularly as well as on the cell surface.

Expression of ADAM15 in the ovarian cancer cell line OV-MZ-6 was verified in the same manner as with the CHO cells.

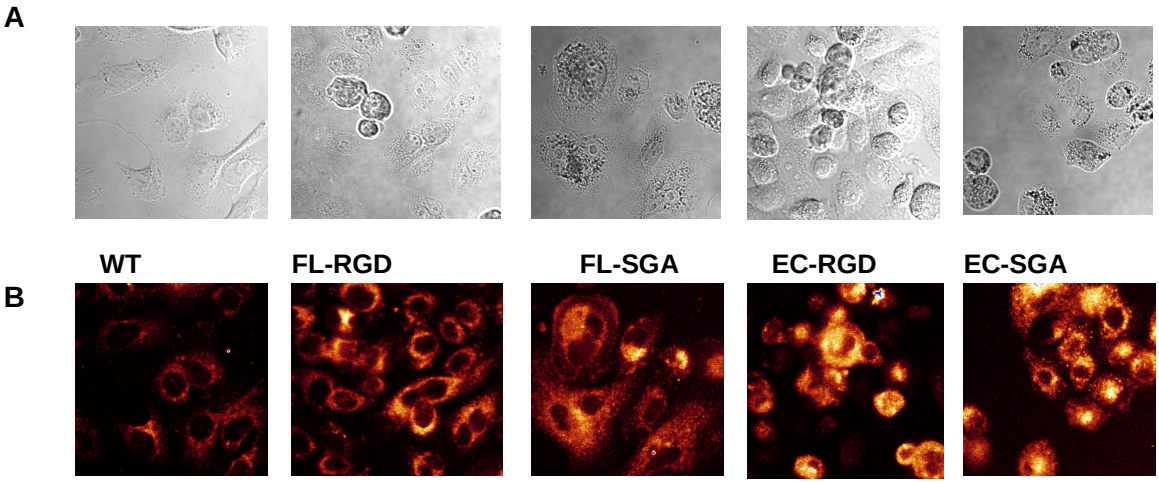


Figure 10 Immunostaining (CLSM) of the expression of human ADAM15 in stably transfected OV-MZ-6 cells
A: Transmission; **B**: Fluorescence; (pictures kindly provided by PD Dr. U. Reuning)
Primary antibody: anti-Myc (1 ug/ml)
Secondary antibody: anti-mouse IgG-Alexa-488 (1 ug/ml)

The results of the immunostaining of the OV-MZ-6 cells with the antibody directed to the c-myc epitope were further confirmed using a polyclonal antibody directed to the extracellular domain of ADAM15.

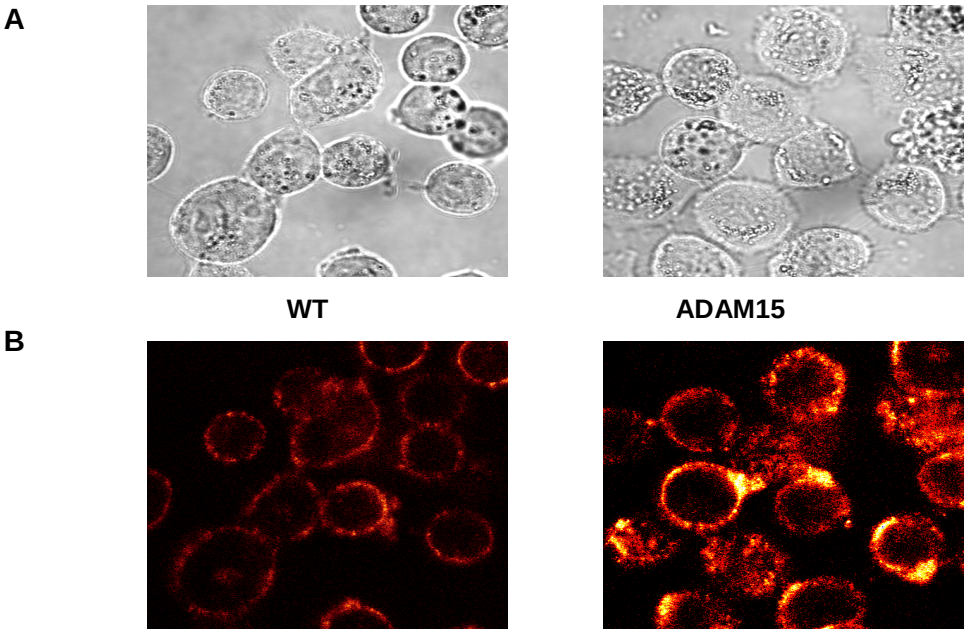


Figure 11 Immunostaining (CLSM) of the expression of human ADAM15 in stably transfected OV-MZ-6 cells
A: Transmission; **B**: Fluorescence; (pictures kindly provided by PD Dr. U. Reuning)
Primary antibody: anti-ectodomain (anti serum 1:500). Secondary antibody: anti-rabbit IgG-Alexa-568 (1 ug/ml)

Staining with the anti-ectodomain antibody allowed detection of both the endogenously expressed ADAM15 in the OV-MZ-6 WT cells and of the overexpression of the ADAM15 protein in the ADAM15 transfected cells.

2. 2 Materials

Instruments

Titertek Multiscan MCC/340 - ELISA Reader	Labsystems, Egelsbach, Germany
Eppendorf centrifuge 5417 C and 5402	Eppendorf, Hamburg, Germany
Heraeus Biofuge Fresco	Heraeus, Munich, Germany
Beckman DU 640 Spectrophotometer	Beckman, Fullerton, CA, USA
Agarose-Gelapparatus LKB GNA 100	Pharmacia, LKB, Freiburg, Germany
Biometra TI 1 UV-shield	Biometra, Göttingen, Germany
Gel apparatus Miniprotean II	Beckton-Dickinson, Heidelberg, Germany
DNA- Ge lchamber GNA 100	Pharmacia, LKB, Freiburg, Germany
Savant Speed Vac SVC 100	Thermo Quest, Ebelsbach, Germany
PTC-150 Minicycler	Bio-Rad Laboratories, CA, USA
Perkin Elmer Gene Amp PCR System 2400	Perkin Elmer European Life Science C Langen, Germany
β-Counter 1219 Rackbeta	Berthold, Bad Wildbad, Germany
Harvester 96 with vacuum pump, Microsealer 021	TomTec, Orange, CN, USA
1450 Microbeta Plus Liquid and Scintillation Counter	Perkin Elmer Wallac GmbH, Turku, Finland
Phosphor Imager 445SI	Molecular Dynamics, Sunnyvale, USA
Microscope Axiovert 35	Zeiss, Göttingen, Germany
Microchamber slides	Nunc Lab-Tec, IL, USA
Scion Image Software	Scion Corporation, ML, USA

Reagents and materials for molecular biology

RNA isolation kit, “RNA Clean”	TM Systems, AGS, Heidelberg, Germany
Acetyl-CoA, Hyperfilm M (RPN 2106), MicroSpin Rainbow	Amersham Pharmacia Biotech, Bedford, USA
Restriction enzymes, "complete" protease inhibitor cocktail tablets, RNAse-free DNAse I, "Expand High Fidelity"-Polymerase	Roche Diagnostics, Mannheim, Germany
Human FN, human VN, human LN, human Col	Collaborative Biomedical Products, Bedford, Ma, USA
X-OMAT AR films	Kodak, Rochester, MN, USA
Nucleobond AX-DNA Isolation Kit	Macherey-Nagel, Düren, Germany
Nitrocellulose Opititran BA-S85 0,45	Schleicher & Schuell, Dassel, Germany
[^{3}H]-thymidine	ICN, Eschwege, Germany
Poly-L-Lysine (PL)	Sigma, Deisenhofen, Germany
BCA Protein Reagent Kit	Pierce, Rockford, USA
ECL-Western Blotting System™	Amersham, NJ, USA
Hexosaminidase reagent (p-nitrophenyl-N-acetyl-beta-D-glucosaminide)	Sigma, Deisenhofen, Germany
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide thiazolyl blue	Sigma, Deisenhofen, Germany

Paraformaldehyde (PFA)	Sigma, Deisenhofen, Germany
Oligodeoxynucleotides	Metabion, Martinsried, Germany

General solutions and buffers

SOLUTION/BUFFER	CONCENTRATION	INGREDIENTS
50x TAE for agarose gels	2.0 M 0.5 M 2 mM pH 7.9	Tris/HCl Na-acetate **E**thylene **d**iamine **t**etraacetic **a**cid (EDTA)
Ethidiumbromide staining solution	10 mg/ml in H_20	Ethidiumbromide
Protein-application buffer (3x) **s**odium**d**odecyl**s**ulfat (SDS) gel electrophore	15 mM 15 % (w/v) 45 % (v/v) 0.1 % (w/v) 6 % (v/v)	Tris/HCl, pH 6.8 SDS Glycerol Bromphenolblue β-mercaptoethanol
Running buffer (10x) for SDS gel electrophoresis	0.25 M 1.95 M 1 % (w/v) pH 8.8	Tris/HCl glycine SDS
TE	10 mM 0.1 mM	Tris/HCl, pH 8.0 EDTA
Phosphate buffer saline PBS	137 mM 2.7 mM 7.3 mM 1.47 mM	NaCl KCl Na_2HPO_4 KH_2PO_4

Antibodies

Monoclonal mouse IgG directed against human c-myc	Invitrogen, Leek, The Netherlands
Polyclonal hamster IgG directed against mouse, Alexa 488 conjugated	A-11029, Molecular Probes, Eugene, OR, USA
Rabbit IgG antibody directed against the extracellular domain of human ADAM15	provided by Carl Blobel, New York, USA

Cell culture reagents

Dulbecco's Modified Eagle Medium (DMEM)	Gibco BRL Life Technology, Eggenstein, Germany
Fetal calf serum (FCS)	Gibco BRL Life Technology, Eggenstein, Germany
Antibiotics (Penicillin/Streptomycin), EDTA -1% (w/v) and BSA	Seromed Biochrom KG, Berlin, Germany
Geneticine (G-418-sulfate)	Calbiochem Novabiochem, Bad Soden, Germany
L-arginine und L-asparagine	Sigma-Aldrich GmbH, Steinheim, Germany

2.3 Methods

2.3.1 Cell culture

Cells were cultivated in cell culture flasks in an incubator at 37 °C/5 % (v/v) CO_2 and passaged every 3-4 d. After washing of the cells in sterile PBS, cells were detached in PBS 0.02 % (w/v) EDTA solution. Then, cells were centrifuged at 1,500 x g for 5 min. The cell pellet was resuspended in fresh medium and passed to a new cell culture flask.

Freezing of cells

5×10^6 cells were detached as described, centrifuged and resuspended in freezing-medium (90 % (v/v) FCS; 10 % (v/v) dimethyl sulfoxide (DMSO). After storage at -80 °C for 3-4 d, cells were transferred to liquid nitrogen (-196 °C).

Medium composition for cell culture

Complete Medium for CHO cells and OV-MZ-6 cells:

- DMEM with Glutamax I
- 10 mM HEPES
- 550 mM arginine
- 272 mM asparagine
- 1000 U/l Penicillin/Streptomycin
- 10 % (v/v) fetal calf serum (FCS)

Selection medium for stably transfected CHO and OV-MZ-6 cells:

- Complete medium supplemented with G 418 at a final concentration of 1g/l

Coating of cell culture dishes with ECM proteins

Coating of cell culture plastic surfaces was carried out with different ECM proteins, diluted in PBS to the following concentrations.

- LN 5 µg/ml
- FN 10 µg/ml
- Col I 5 µg/ml
- Col IV 5 µg/ml
- PL 0.01 % (v/v)
- VN 10 µg/ml

Coating was conducted at 4 °C overnight. After subsequent washing of the dishes in sterile PBS, uncoated cell culture areas were blocked with 1.5 % (w/v) heat-denaturated, sterile BSA in PBS at room temperature for 2 h prior to cell seeding.

Generation of cell lysates

Cells were washed in cold PBS, incubated with a lysis buffer on ice and then harvested using a cell scraper. The lysates were centrifuged at 14,000 x g at 4 °C for 10 min and the supernatants conveyed to new reaction tubes.

Lysis buffer:

- 50 mM EPES, pH 7.5
- 150 mM NaCl
- 1 mM EDTA
- 10 % (v/v) glycerole
- 1 % (v/v) Triton X-100

Freshly added:

- 10 mM NaF
- 1 mM Na-orthovanadate
- 10 µg/ml aprotinine
- 1 mM phenylmethylsulfonylfluoride

2.3.2 Isolation of total RNA

RNA was isolated according to the guanidinium-phenol-chloroform-method described by Chomczynski and Sacchi (1987).
2 x 10^6 cells were plated in 10 cm cell culture petri dishes and incubated till the cells reached approximately 50 % - 60 % confluency. Cells were then rinsed with cold PBS. Then "RNA-Clean"-solution was added and the cells collected in a reaction tube. After addition of 10 % of the total volume of chloroform it was centrifuged at 10,000 x g at 4°C. The upper aqueous phase was removed, and then the total RNA precipitated using an equal volume of isopropanol (purity 260/280). After second centrifugation and two-time washing with 70 % (v/v) ethanol (EtHOH), the precipitated RNA was solubilized in RNAse-free water. The concentration was spectrophotometrically determined at an absorbance of [A = 260 nm].

2.3.3 Determination of protein concentrations

The determination of protein concentrations was carried out using the "BCA Protein Assay Reagent"-Kit (Pierce, Rockford, USA). This test is based on the reduction of Cu^{2+} to Cu^{1+} by the protein in an alkaline medium (Biuret reaction, Smith et al., 1985). Due to this reaction, a colored complex is being generated between Cu and the BCA-protein complex. For the establishment of a standard curve, BSA in increasing concentrations between 20 µg/ml and 400 µg/ml was used. After application to a 96-well of the standard, sample, or buffer (control), 200 µl of the BCA reagent were added. Incubation was for 2 h at 37 °C. Absorption was measured in an ELISA reader at an absorbance of [A = 560 nm]

2.3.4 Reverse-transcription polymerase chain reaction

In order to verify successful transfection of OV-MZ-6 and CHO cells with ADAM15 on the RNA level, **R**everse-**T**ranscription **P**olymerase **C**hain **R**eaction (RT-PCR) was performed.
First, total RNA, pretreated with DNAse I was reverse described into cDNA. 3-5 µg of total RNA was used in each RT-reaction and inserted into a reaction tube. Both 1 µl of DNAse and DNAse buffer (10 x) were added to the RNA. RNAse-free water was then added ad 10 µl. The mix was incubated for 15 min at RT. Afterwards, 1 µl of EDTA was added and the tube was incubated for another 10 min. Next, 1 µl of oligo dT primers (2.5 mM) was added and the mix incubated for further 5 min. Then, on ice, 5 µl synthesis buffer (5x), 1 µl (desoxy-nucleoptide-triphosphate) dNTPs (10 mM), 2 µl dithiothreitol DTT (100 mM), 1 µl Superscript Reverse Transcriptase, and 1 µl RNAsin (RNAse inhibitor) were added and the reaction tube was mixed thoroughly. After incubation at 42 ºC for 50 min, at 90 ºC for 5 min,

and on ice for 10 min, 1 µl of RNAse H was added and the tube incubated for another 20 min at 37ºC. The cDNA was frozen at -20 ºC till use in PCR reactions.

RT-PCR analysis for the determination of ADAM15 mRNA concentrations was conducted using the following primers: 5´ -GGC TGG CAG TGT CGT CCT ACC AGA GGG G-3´ and 5´-GGT GCA CCC AGC TGC AGT TCA GCT CAG TCC-3´ (amplicon size 420 bp). In order to ensure equal RNA concentrations and efficiency of the RT reactions, the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in the same PCR reaction using the following primers: GAPDH 5´: 5´-CCA TGG AGA AGG CTG GGG-3´ and GAPDH-3´ : 5´-CAA AGT TGT CAT CGA TGA CC-3´ (amplicon size 194 bp).

For the PCR reaction, 4 µl of cDNA generated in the RT reaction was used. Working on ice, a PCR "master mix" was created, using 5 µl of buffer, 5 µl of dNTPs (2.5 mM), 4 µl of cDNA, 3 µl each of GAPDH 3' primer, GAPDH 5' primer, ADAM15 3' primer, and ADAM15 5' primer (each at 20 pmol/µl). 23 µl of dH_2O were added ad 50 µl. 1 µl of High-Fidelity Tag polymerase was added to each reaction tube.

The cycle used was: 1 cycle of 5 min at 94 ºC. This was followed by 35 cycles of: 60 sec denaturation at 94 ºC, 1 min annealing at 50 ºC, and 90 sec extension at 72 ºC. PCR products were electrophoresed on Trisborate-EDTA-polyacrylamide gels. Signal intensity was evaluated by densiometrical scan using the *Scion Image Software* with data presented as ratio of ADAM15 to GAPDH amplicons.

2.3.5 Western blot analysis

Proteins were separated on 10 % (w/v) SDS-gels and blotted to a nitrocellulose membrane in a wet blot chamber at 25 V overnight. Blocking of free binding sites of the membrane was conducted with 1x NET gelatine at room temperature for 2 h. After 3 washes in 1 x NET gelatine, the diluted, peroxidase-coupled second antibody was added and incubated for 1 h. After renewed washing, the membrane was incubated with ECL-Western Blotting System™ luminescent reagent for 5 min. Signals were detected by exposing the nitrocellulose membrane to X-ray films for a period ranging from 5 sec to 1 h.

10 x NET-Buffer:

- 1.5 M NaCl
- 0.05 M EDTA
- 0.5 M Tris/HCl
- 0.1 % (v/v) Triton X-100

1 x NET-Gelatine:

- 100 ml 10 x NET-buffer
- 0.25 % (w/v) gelatine
  ad 1 l with dH_2O

2.3.6 SDS-polyacrylamid gel electrophoresis

Vertical gels (SDS-Page, Laemmli 1970) are comprised of a 4 % (w/v) polyacrylamide collecting gel and a 7.5 % or 10 % (w/v) separating gel. Cell lysates were added to sample buffer and boiled at 100 °C for 5 min prior to application to the gel. Electrophoresis was conducted in a minigel apparatus at 120 V/RT.

Collecting gel:

- 0.125 M Tris/HCl, pH 6.8
- 4 % (w/v) acrylamid
- 0.11 % (w/v) bisacrylamid
- 0.1 % (w/v) SDS
- 0.054 % (w/v) $H_8N_2O_8S_2$
- 0.42 % (v/v) TEMED

Separating gel:

- 0.4 M Tris/HCl pH 8.8
- 7.5 or 10 % (w/v) acrylamide
- 0.18 % (w/v) bisacrylamide
- 0.1 % (w/v) SDS
- 0.05 % (w/v) $H_8N_2O_8S_2$
- 0.04 % (v/v) TEMED

2.3.7 Cell adhesion assay

As a measure of determining the number of adherent cells, the direct proportionality between the number of cells and the amount of the ubiquitously distributed lysosomal enzyme, N-acetyl-ß-D-hexosaminidase has been used (adapted from Landegren, 1984).
Hexosaminidase (2-acetamino-2-deoxy-ß-D-glucoside-beta-amidodeoxygluco-hydrolase) in cells is active as it degrades glycosylated cell components. Hereby, a chromogenic substrate (p-nitrophenol-N-acetyl-ß-D-glucosamide) is used, which is degraded by hexosaminidase. The products are measured in an ELISA reader at an absorbance of [A =

405 nm]. 96-well flat-bottomed microtiterplates were coated with different ECM proteins and incubated at 4 °C overnight. After washing the plates in sterile PBS, free binding sites on the plates were blocked using 1.5 % (w/v) heat-denaturated, sterile BSA at RT for 2 h. Cells were rinsed and detached from the flasks as described before in chapter 2.3.1. After centrifugation at 1,000 x g, cells were resuspended in cell culture medium and counted in a Neubauer hemocytometer.

Precoated 96-wells were rinsed three times and cells plated at a cell density of 3 x 10^4 cells/well. A cell-free well in PBS served as a control. After distinct time intervals of cell cultivation, non-adherent cells were removed by two washes in 37 °C PBS. 50 µl of hexosaminidase substrate per well were then added and the reaction terminated after 1 h of incubation at 37 °C/5% (v/v) CO_2 with stop-buffer. The amount of enzymatically processed substrate was measured at an absorbance of [A = 405 nm] in an ELISA plate reader. Measurements were conducted by triplicate determinations, and for the determination of cell numbers, a standard curve was established.

Adhesion medium:

- DMEM
- 0.5 % (w/v) BSA
- 20 mM HEPES

Hexosaminidase substrate:

- 100 mM Na-citrate; pH 5,0
- 0.5 % (v/v) Triton X-100
- 15 mM p-nitrophenyl-N-acetyl-beta-D-glucosaminide

Stop buffer:

- 0.2 M NaOH
- 5 mM EDTA

2.3.8 Determination of cell proliferation

Cell counting

After coating of 6-well flat-bottom microplates with VN (10 µg/ml), 6,000 cells/well of each cell type were plated and incubated at 37 °C/5% (v/v) CO_2 for different periods of time, ranging from 24 h to 96 h. Cells were detached from the wells as described and counted in a Neubauer hemocytometer upon trypan blue exclusion.

MTT tests

The reduction of tetrazolinum 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) to a blue formazan product by cellular mitochondrial dehydrogenases is used to determine cell numbers (adapted from Carmichael et al., 1987, 268-76). This is based on linear correlation of enzymatic activity of mitochondrial dehydrogenases to cell numbers.
96-well flat-bottom microplates were coated with different ECM proteins as described. Cells were plated at a density of 5,000 cells/well. After incubation at 37 °C/5% (v/v) CO_2 for different periods of time of up to to 96 h, MTT was added in a concentration of 200 µg/ml per well. After 2 h of incubation at 37 °C/5% (v/v) CO_2, the medium was removed and the generated purple crystals dried and dissolved in 50 µl of DMSO. Measuring was performed at an absorbance of [A= 590 nm] in a Titertek Multiscan Elisa plate reader. The absorption measured was proportional to the number of cells.

Measurement of the *de novo* DNA synthesis rate by [^{3}H]-thymidine incorporation

The rate of *de novo* DNA synthesis was determined by measuring the uptake of [^{3}H]-thymidine into cellular DNA.
96-well flat-bottom microplates were precoated with different purified ECM proteins, blocked with PBS 2% (w/v) BSA and rinsed with PBS as described. Then, 2 x 10^4 cells/well were seeded into each well and incubated at 37 °C/5% (v/v) CO_2 for distinct time intervals of up to 96 h. After addition of [^{3}H]-methyl thymidine (25 µCi/ml) to each well and subsequent incubation for further 6 h, cell culture plates were frozen. The defrosted samples were harvested using the Harvester 96. Hereby, for each plate a glasfiber filter was put into the Harvester and each cell suspension was then sucked through one square of the filter into the Harvester. A new filter was used for each plate and the Harvester rinsed 3 times afterwards. Each filter was placed into a plastic bag together with a wax platelet. The wax was then melted onto the filter in a Microsealer 1495-021 and cell-associated radioactivity for each sample counted in a 1450 Microbeta Plus Liquid Scintillation Counter and

expressed as ***c***ounts ***p***er ***m***inute (cpm). In parallel, cells were plated into identically coated plates and lysed. In order to normalize the amount of cell-associated radioactivity to cell numbers, either cell counting was performed in parallel wells or protein concentrations determined as measure of cell numbers. The value of the incorporated [^{3}H]-thymidine was then set into relation to the amount of protein or the number of cells.

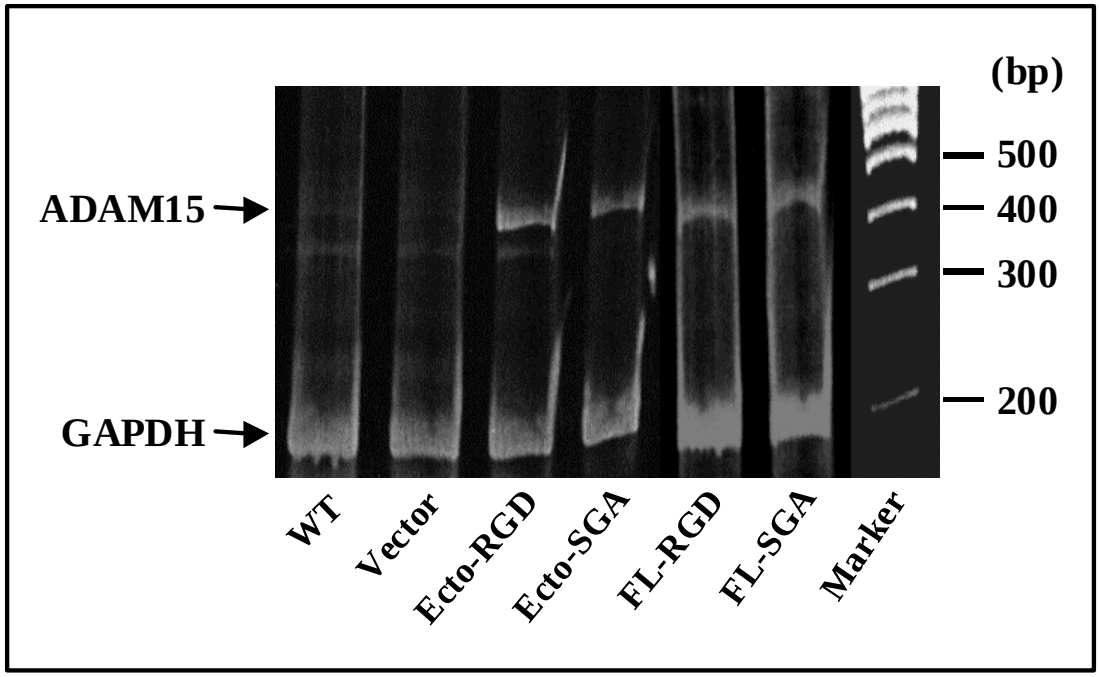
3. RESULTS

3.1 Determination of ADAM15 expression in stably transfected OV-MZ-6 cells using RT-PCR

In order to determine ADAM15 transcript concentrations in the OV-MZ-6 cells after transfection, semi-quantitative RT-PCR was performed on total isolated RNA, as described before. Amplification of a housekeeping gene, the GAPDH, was used as a control for the efficiency of the RT-PCR reaction. Depicted in the following is a representative RT-PCR experiment, which was conducted three times. It shows the signal intensity of the ADAM15 and GAPDH amplicon generated within the same PCR reaction tube.

The ADAM15/GAPDH ratio of the transfected cell clones of each transfection category is markedly elevated when compared to the WT and vector-transfected control cell clones. The ratio of ADAM15/GAPDH expression in the A-15 FL cell clones was higher than in the A-15 EC cell clones (A-15 FL-RGD #1: 4.7-fold; A-15 FL-SGA #1: 2.5-fold; A-15 EC-RGD #1: 1.9-fold; A-15 EC-SGA #1: 1.8-fold). The significantly higher ratio of ADAM15/GAPDH mRNA in the ADAM15 transfected cells indicates a successful transfection and expression of the ADAM15 transcripts.

A



B

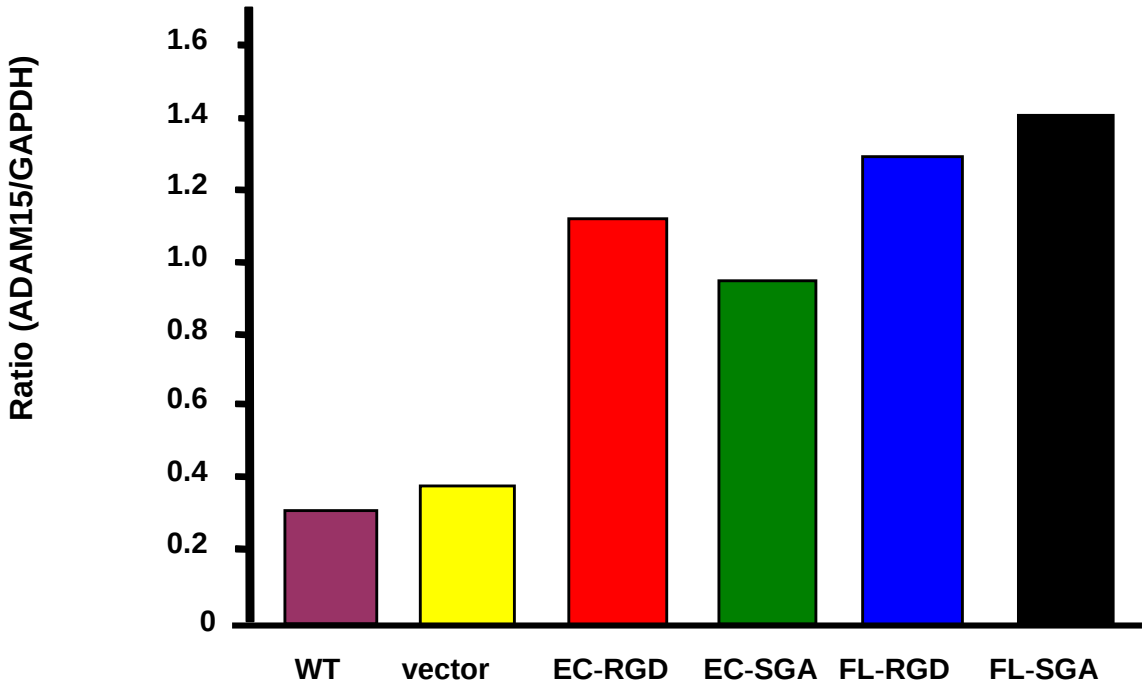


Figure 12 A/B Semi-quantitive RT-PCR analysis of ADAM15 mRNA concentrations after OV-MZ-6 cell transfections with ADAM15 cDNAs

Total RNA of WT, vector- or ADAM15-transfected OV-MZ-6 cells were harvested and RT-PCR analyses conducted, as described earlier. ADAM15 amplification products were electrophoresed on Tris-borate-EDTA-polyacrylamide gels. As control served GAPDH, which was amplified in the same PCR reaction tube. A representative experiment is depicted, which was further evaluated for signal intensity by densitometrical scan using the *Scion Image Software*. Expression of ADAM15 mRNA in all ADAM15 transfection categories as indicated by the ADAM15/GAPDH mRNA ratios is elevated when compared to the WT and vector-transfected cell clones. ADAM15/GAPDH mRNA ratios in the *FL* ADAM15 cell clones are higher than in the *EC* ADAM15 cell clones.

3.2 Effect of ADAM15 overexpression on the adhesive phenotype of the human ovarian cancer cell line OV-MZ-6 measured in adhesion assays

Ovarian cancer cells organize their adhesion to the ECM component VN via interaction between integrin αvβ3 and the amino acid triplet RGD within VN (Hapke et al., 2001, 26340-48). ADAM15 possesses an RGD motif in its disintegrin domain and, upon overexpression in OV-MZ-6 cells, could function as a natural competitor for VN binding to integrin αvβ3, thereby possibly changing cell adhesion properties.

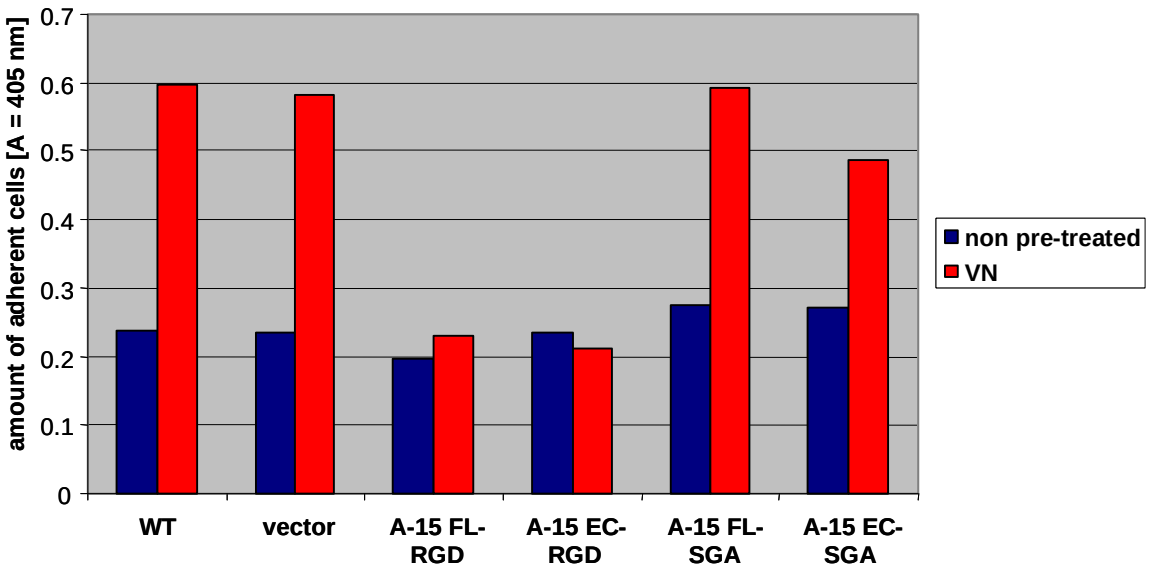


Figure 13 Adhesion of ADAM15 overexpressing OV-MZ-6 cells to non pre-treated cell culture plates and purified VN as growth substrate [A = 405 nm]
Adhesion assays were performed as described. 35,000 cells were plated in either VN-coated or non pre-treated 96-well cell culture plates and incubated for 90 minutes. Adhesion of the
A-15 FL-RGD overexpressing OV-MZ-6 cells and the A-15 EC-RGD cell clones to VN was markedly reduced when compared to WT, vector-transfected, and A-15 FL-SGA mutant control cell clones. (n = 3) (SD depicted in table 2).

Up to three alternative and independently isolated OV-MZ-6 cell clones of each transfection category with similar ADAM15 expression levels were tested. Adhesion assays with ADAM15 overexpressing OV-MZ-6 cells to VN-coated 96-well cell culture plates revealed a significant reduction of the adhesion of A-15 FL-RGD and A-15 EC-RGD overexpressing OV-MZ-6 cell clones to VN when compared to WT and vector-transfected cell clones. The A15 SGA-mutant control cell clones behaved like WT and vector-transfected cells. Adhesion of the A-15 FL-RGD cell clones to VN, when compared with WT cells was reduced by 39 %. Adhesion of the same cell clones to VN when compared with the A-15 FL-SGA mutant

control cell clones was reduced by 39 %. ADAM15 EC-RGD overexpression led to a decrease in adhesion to VN of 35 % when compared with WT cells, and of 36 % when compared with vector-transfected cell clones.

Cell type (OV-MZ-6)				
ECM	**non pre-treated**	**SD**	**VN**	**SD**
WT	0.237	0.007	0.598	0.011
vector	0.236	0.008	0.583	0.088
A-15 FL-RGD	0.196	0.008	0.231	0.01
A-15 EC-RGD	0.235	0.013	0.211	0.01
A-15 FL-SGA	0.275	0.009	0.591	0.01
A-15 EC-SGA	0.272	0.004	0.486	0.01

Table 2 Adhesion of ADAM15 overexpressing OV-MZ-6 cell clones to non pre-treated cell culture plates and purified VN as depicted in figure 13 [A = 405 nm]
Adhesion assays were performed by chromogenic assays and subsequent spectrophoto-metrical measurements using either non pre-treated cell culture dishes or cell culture dishes coated with the purified ECM protein VN. Depicted are mean values and standard deviations (+/-SD) of three independent determinations.

In order to further characterize the adhesive behavior of ADAM15 ecto-domain overexpressing cell clones, adhesion assays with A-15 EC-RGD cell clones were performed. A-15 EC-SGA mutant cell clones served as controls, in addition to WT and vector-transfected cell clones.

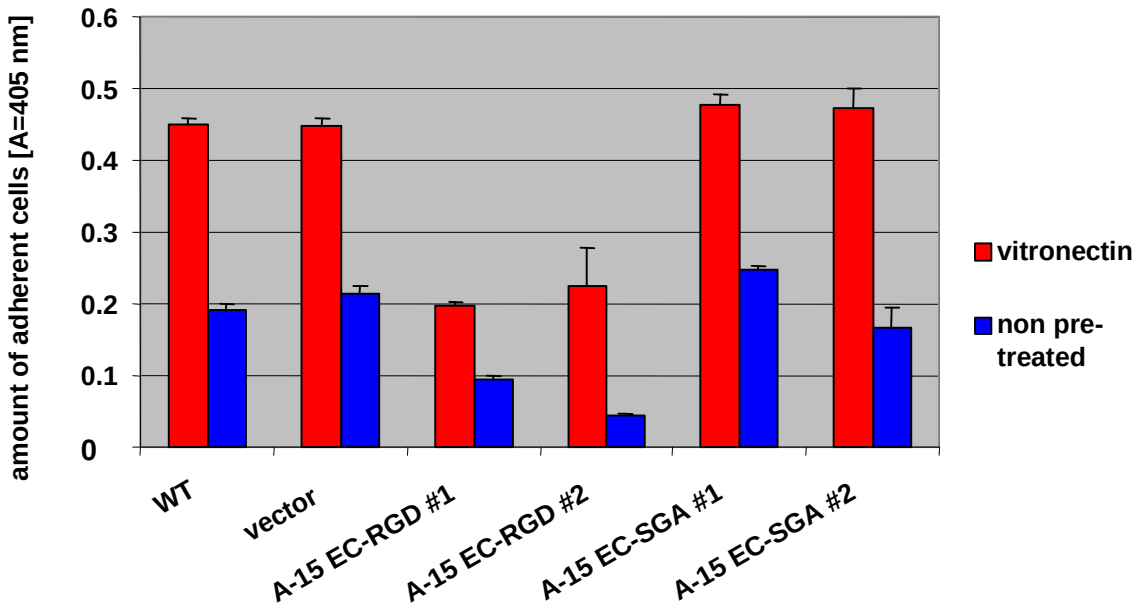


Figure 14 Adhesion of ADAM15 ecto domain overexpressing OV-MZ-6 cell clones to non pre-treated cell culture plates and purified VN [A = 405 nm]
The adhesion assays were conducted as described previously. Adhesion of the A-15 EC-RGD overexpressing OV-MZ-6 cell clones to VN is markedly reduced when compared to the A-15 EC-SGA mutant control cell clones, and the WT and vector-transfected control cell clones. Adhesion of the A-15 EC-RGD overexpressing cells to an uncoated cell culture surface was also reduced when compared to control cell clones (n = 3).+/- SD

Adhesion of A-15 EC-RGD overexpressing cell clones to VN was significantly reduced when compared with WT, vector-transfected, and A-15 EC-SGA mutant control cell clones. Adhesion of the A-15 EC-RGD #1 and #2 cell clones to VN was reduced by 56 % and 59 %, respectively, when compared to WT cells. Adhesion of the A-15 EC-RGD cell clones #1 and #2 was reduced by 55 % and 53 %, respectively, when compared to the A-15 EC-SGA #1 mutant cell clones.

The effect of the ECM proteins Col I + IV, which mainly bind to integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ on the adhesive behavior of ADAM15 overexpressing OV-MZ-6 cells was examined in adhesion assays using 96-well cell culture plates pre-treated with Col I and Col IV, respectively. As shown in our previous experiments, adhesion of A-15 FL-RGD cell clones to VN was significantly reduced when compared to WT, vector-transfected, and A-15 FL-SGA mutant control cell clones, respectively. There was no marked decrease of adhesion of the A-15 FL-RGD cell clones on *non-pretreated* plastic growth surfaces when compared to WT, vector-transfected and A-15 FL-SGA mutant control cell clones. Adhesion of ADAM15-FL-RGD overexpressing cell clones to VN was decreased by 41 % when compared to WT, by 46 % when compared to the vector-transfected cell clones, and by 48 % when compared to the A-15 FL-SGA mutant control cell clones. This indicates the potential competitive inhibition of cell adhesion to VN by the ADAM15-RGD overexpressing OV-MZ-6 cells.

Adhesion levels of all cells tested to Col I were found to be significantly lower compared to adhesion on Col IV-treated surfaces. Adhesion of A-15 FL-RGD cells to Col I-coated surface showed no marked reduction of adhesion compared to the WT, vector-transfected, and the A-15 FL-SGA mutant control cell clones, respectively.

Adhesion of all cells to Col IV was found to be stronger as has been shown before by Hapke et al (2001). However, there was no significant reduction of the adhesion of the A-15 FL-RGD cells when compared with WT, vector-transfected, and the A-15 FL-SGA mutant control cell clones.

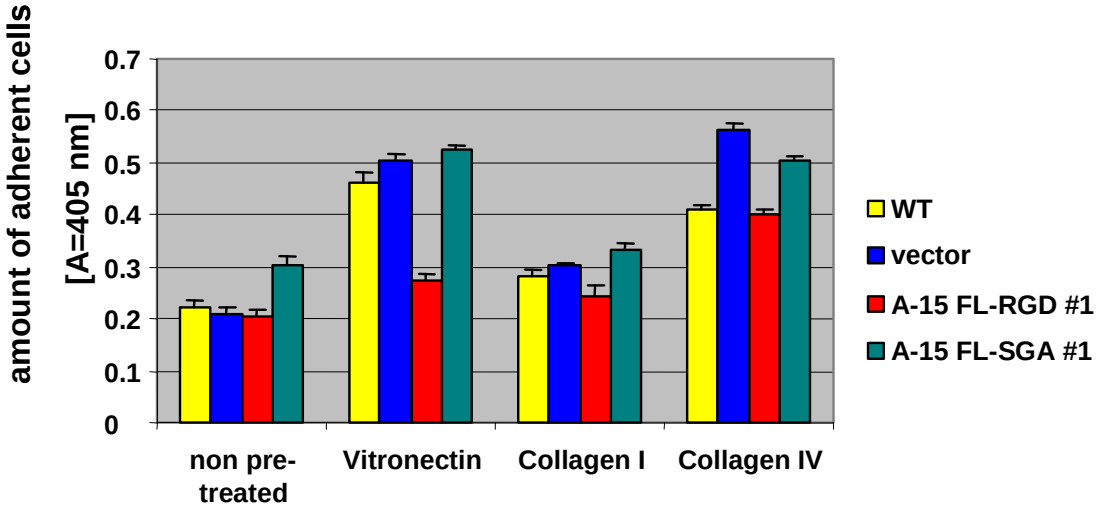


Figure 15 Adhesion of ADAM15 overexpressing OV-MZ-6 cells to different ECM proteins
Adhesion assays were carried out as described. 35,000 cells were incubated on the respective purified ECM proteins for 90 min. Adhesion of A-15 FL-RGD overexpressing OV-MZ-6 cell clones to VN was markedly reduced when compared with WT, vector-transfected, and A-15 FL-SGA mutant control cell clones. Adhesion of the A-15 FL-RGD cell clones to the non-pretreated plates was not reduced when compared to control cells. Adhesion of the ADAM15-RGD overexpressing cell clones to the Col I-coated plates did not show a significant decrease in adhesion. Adhesion to the Col IV-coated cell growth surface was generally stronger for all cell clones tested compared to adhesion to Col I. There was no significant reduction of adhesion of the A-15 FL-RGD cell clones when compared with the control cell clones (n = 3).+/- SD

Investigating the adhesive behavior of the A-15 EC cell clones to different purified ECM proteins, the A-15 EC-RGD cell clones showed a reduced adhesion to VN when compared with WT, vector-transfected, and the A-15 EC-SGA mutant control cell clones. Adhesive strength of all cells to Col I was lower than to VN. There was no reduction of the adhesion of the A-15 EC-RGD cells to the non pre-treated surface indicating no competitive impact of the EC-RGD-motif on this surface.

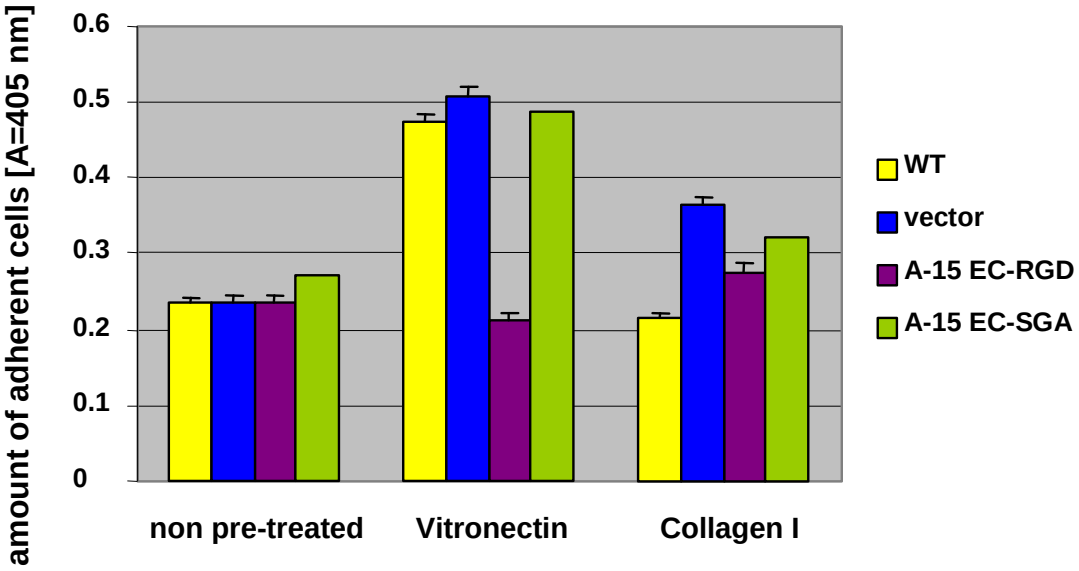


Figure 16 Adhesion of ADAM15 ecto-domain overexpressing OV-MZ-6 cells to different purified ECM proteins

Adhesion assays were conducted as described. The ADAM15 EC-RGD overexpressing cell clones show a markedly reduced adhesion to VN when compared to WT, vector-transfected, and ADAM15 EC-SGA mutant control cell clones. Adhesion of ADAM15 EC-RGD cell clones to Col I was not significantly reduced. There was no reduction in the adhesion of the A-15 EC-RGD overexpressing cells to the non pre-treated cell culture dish surface (n = 3). +/- SD

3.3 Effect of ADAM15 expression on the adhesive phenotype of Chinese hamster ovary cells measured in adhesion assays

In order to confirm the effects of ADAM15 on αvβ3-mediated cell adhesion, we employed an alternative cell culture model by stably transfecting CHO cells with the four different ADAM15 cDNA constructs, ADAM15 FL-RGD or EC-RGD and A-15 FL-SGA and A-15 EC-SGA mutant control cell clones.

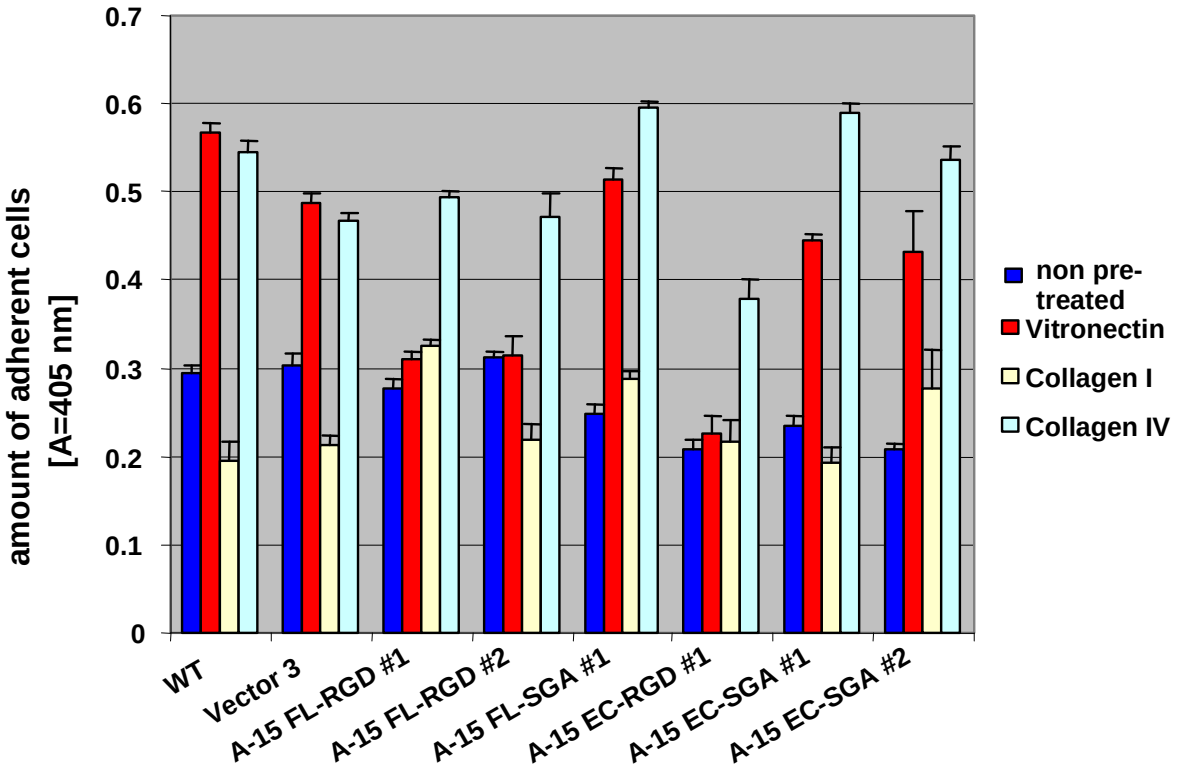


Figure 17 Adhesion of ADAM15-expressing CHO cells to different purified ECM proteins
Adhesion assays were carried out as described. Both ADAM15 FL-RGD #1 and #2 cell clones and A-15 EC-RGD #1 and #2 cell clones show a significantly reduced adhesion to VN when compared to control ADAM15 FL-SGA mutants, WT, and vector-transfected cell clones. Adhesion of the ADAM15 FL-RGD and EC-RGD expressing CHO cell clones to Col I was not significantly reduced when compared to the WT, vector-transfected, and the A-15 FL-SGA mutant control cell clones. Analogously to the OV-MZ-6 cell clones tested, adhesion of all cell clones to Col IV was stronger than to the other purified ECM proteins or the uncoated cell growth surface. There was no significant impact of A-15 FL-RGD and A-15 EC-RGD expression on the adhesion to Col IV. On the uncoated surface, there was no reduction in the adhesion of the A-15 FL-RGD and A-15 EC-RGD cell clones (n = 3). +/- SD

Adhesion of the A-15 FL-RGD #1 and #2 expressing CHO cell clones to VN was significantly reduced when compared to WT, vector-transfected, and A-15 FL-SGA #1 mutant control cells, respectively. There was no significant inhibition of cell adhesion to non-pretreated

surface when using A-15 FL-RGD and A-15 EC-RGD expressing CHO cell clones. Adhesion of A-15 EC-RGD cell clones #1 and #2 to VN was significantly reduced when compared to the WT, vector-transfected and A-15 EC-SGA 1 and 2 mutant control cell clones. Adhesion of ADAM15-RGD cell clones to Col IV was not markedly inhibited. Adhesion to Col I was weaker for all cell clones tested and not affected by the expression of ADAM15 or either variant thereof in CHO cells.

3.4 ADAM15 overexpression of OV-MZ-6 cells cultivated on vitronectin causes time-dependent decrease of numbers of adherent cells

3.4.1 Time-dependent decrease of numbers of adherent ADAM15-overexpressing OV-MZ-6 cells in cell-counting assays

In order to investigate the effect of ADAM15 overexpression on the proliferative behavior of anchorage-dependent OV-MZ-6 cells cultivated on VN, cell-counting assays were conducted over a period of 4 d. 4,000 – 6,000 cells of ADAM15 FL and EC cell clones as well as control cells were seeded on cell culture plates pre-treated with purified VN, as described previously. Depicted here is a representative experiment of four, showing that numbers of adherent A-15 FL and A-15 EC-RGD expressing cell clones were significantly reduced when compared to the A-15 FL-SGA and A-15 EC-SGA expressing cell clones, and WT or vector-transfected cells.

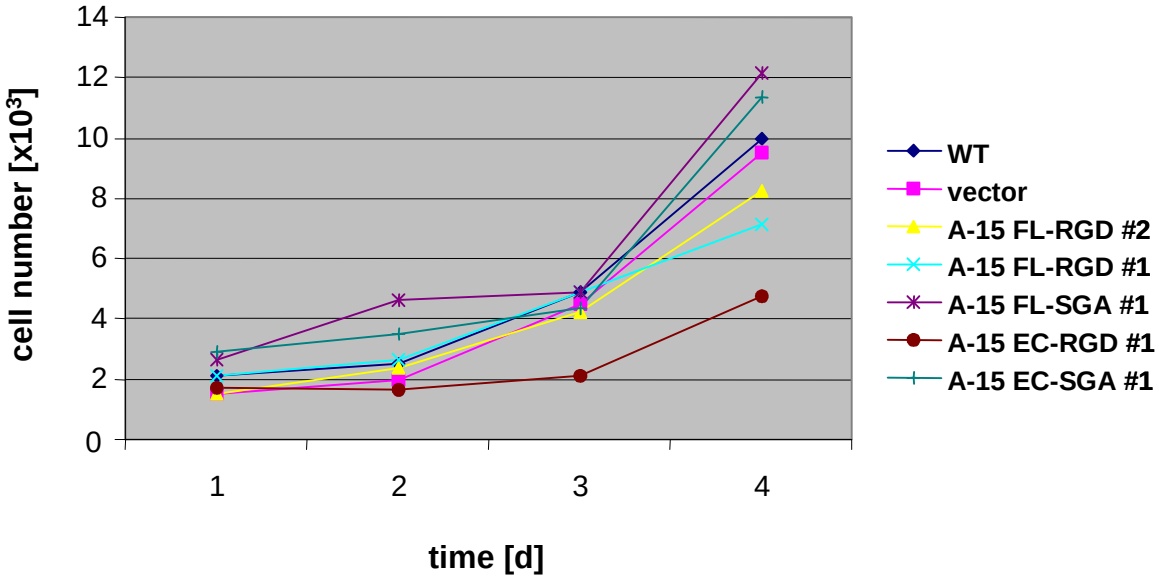


Figure 18 Effect of ADAM15 expression on numbers of adherent OV-MZ-6 cells determined by cell counting over time
Cell counting assay comparing numbers of adherent ADAM15 overexpressing OV-MZ-6 cells on VN as adhesion-supporting matrix. 5,000 cells of each different cell type were plated on VN-coated cell culture plates, and cell numbers were measured each day in a hemocytometer for 4d. A-15 FL-RGD overexpressing cell clones #1 and #2, and the A-15 EC-RGD #1 overexpressing cell clones show a decreased cell numbers when cultivated on VN compared to A-15 FL-SGA #1, A-15 EC-SGA #1control cell clones, and to WT or vector-transfected cell clones.

After 4 d, numbers of adherent cells of A-15 FL-RGD #1 and #2 cell clones were reduced by 32 % and 41 %, respectively when compared to A-15 FL-SGA mutant control cell clones.

Cell numbers of adherent A-15 FL-RGD #1 and #2 cell clones compared to the WT cell clones were reduced by 18 % and 29 % after 4 d, respectively. The decrease in cell numbers of the A-15 EC-RGD #1 cell clone compared to the WT and A-15 EC-SGA #1 control cell clones equalled 53 % and 58 % on day 4 of the experiment, respectively.

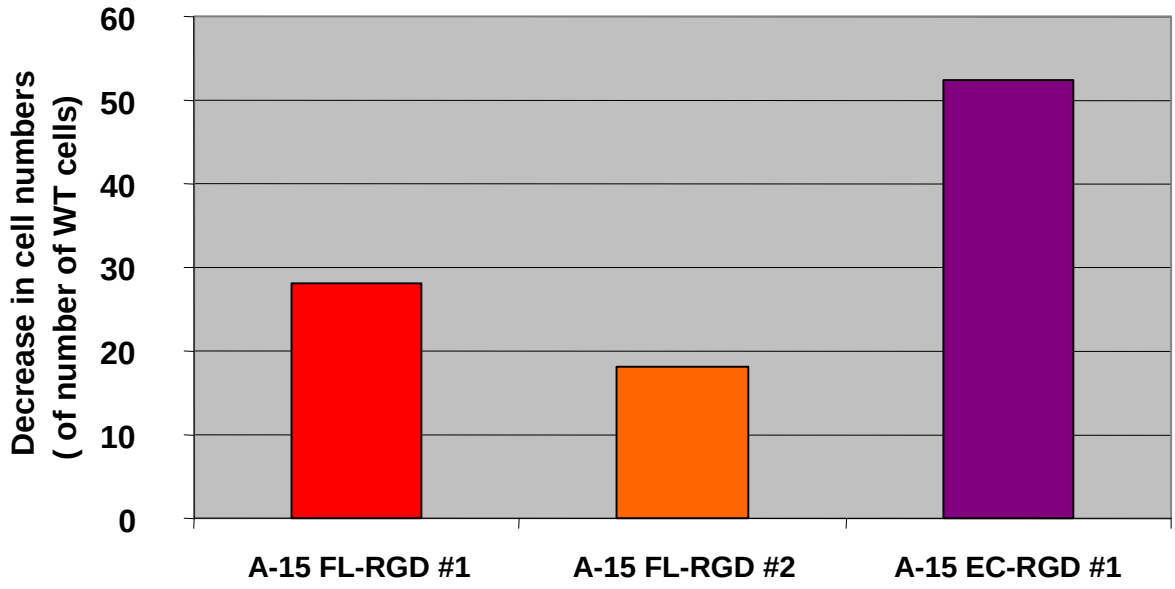


Figure 19 Evaluation of cell numbers of different ADAM15 overexpressing cell clones when compared to WT cell clones on purified VN after 4 d (% of WT cell clones)
Results of the previous cell counting assays expressed as decrease in cell numbers of adherent A-15 FL-RGD and A-15 EC-RGD cell clones when compared to the number of adherent WT cell clones after 4 d. A-15 FL-RGD #1 and #2 and A-15 EC-RGD #1 overexpressing cell clones show a significant decline in cell numbers on VN when compared to the WT cells after 4 d.

As depicted in Fig. 19, cell numbers of adherent A-15 FL-RGD overexpressing OV-MZ-6 cell clones #1 and #2 were reduced by 18 % and 28 %, respectively, when compared to the number of adherent WT cell clones after 4 d. Numbers of adherent A-15 EC-RGD overexpressing ovarian cancer cell clones was reduced by 52 % when compared to WT cells after 4 d. In parallel, cell counting assays were conducted using non pre-treated cell growth surfaces. Depicted in the following is a representative experiment out of four. Numbers of adherent A-15 FL-RGD overexpressing cell clones on VN was reduced by 21 % and 24 % when compared to numbers of adherent WT and vector-transfected clones, respectively, after 4 d. Cell numbers of the A-15 EC-RGD cell clones were reduced by 23 % after 4 d when compared with the A-15 EC-SGA mutant cell clones.

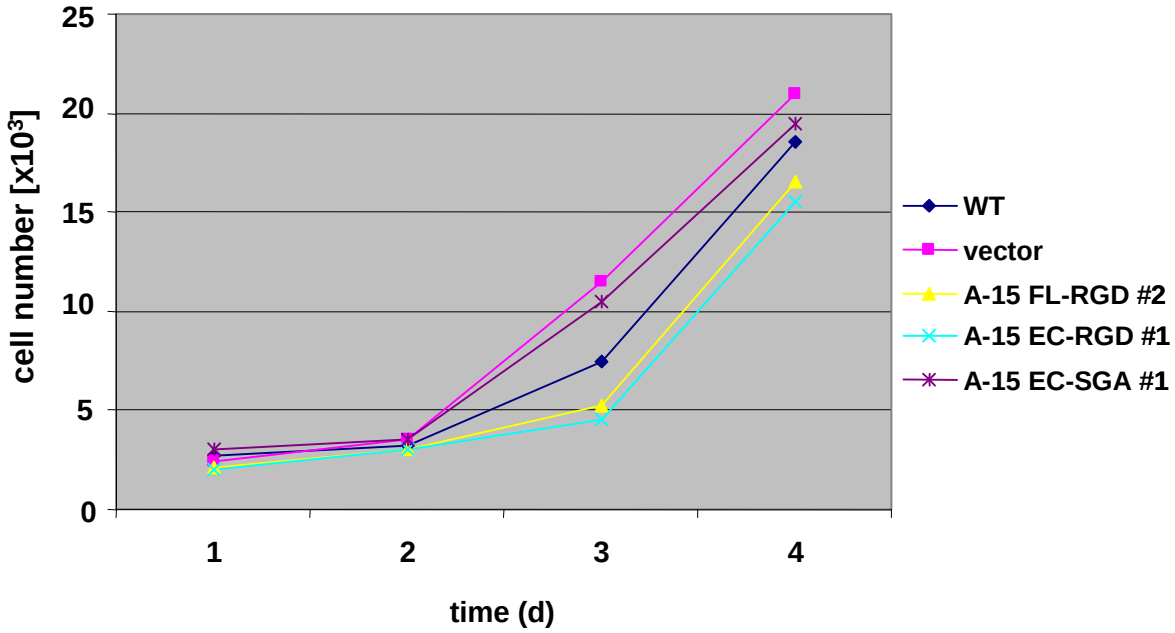


Figure 20 Evaluation of number of adherent ADAM15-overexpressing cell clones on non pre-treated cell growth surfaces over a period of 4 d
Cell counting was performed as described before. 5,000 cells were plated on a non pre-treated cell culture well and incubated for 4 d. Cells were counted at distinct time intervals up to 4 d. Cell numbers of adherent A-15 FL-RGD expresing cell clones and A-15 EC-RGD expressing cell clones were reduced to a smaller degree than when cultivated on VN when compared to the WT and vector-transfected cell clones and the A-15 EC-SGA mutant control #1 cell clones.

On non pre-treated cell growth surfaces, the decrease in numbers of adherent A-15 FL-RGD-overexpressing cells expressed as percent of numbers of adherent WT cell clones was smaller than the decrease on VN-coated plates after 4 d. The decrease in cell numbers of the A-15 EC-RGD overexpressing cell clones was significantly stronger on VN-coated cell culture wells (52 %) than on non pre-treated surfaces (26 %).

3.4.2 Time-dependent evaluation of numbers of adherent ADAM15-overexpressing OV-MZ-6 cells by MTT tests

Alternative to cell counting we determined cell numbers over time by MTT tests as described. For this, cells were plated in a density of 5,000 on VN-coated 96-well cell culture dishes and MTT assays performed. As controls served WT and vector-transfected cell clones.

After 6 d, numbers of adherent A-15 EC-RGD overexpressing cell clones was reduced by 47 % and 22 %, respectively, when compared to numbers of adherent WT cells. Comparing numbers of adherent A-15 EC-RGD overexpressing cell clones to A-15 EC-SGA control cell clones, cell numbers were reduced by 50 % and 27 %, respectively. When compared to the A-15 EC-SGA control cell clones, numbers of adherent A-15 EC-RGD cell clones were diminished by 56 % and 35 %, respectively.

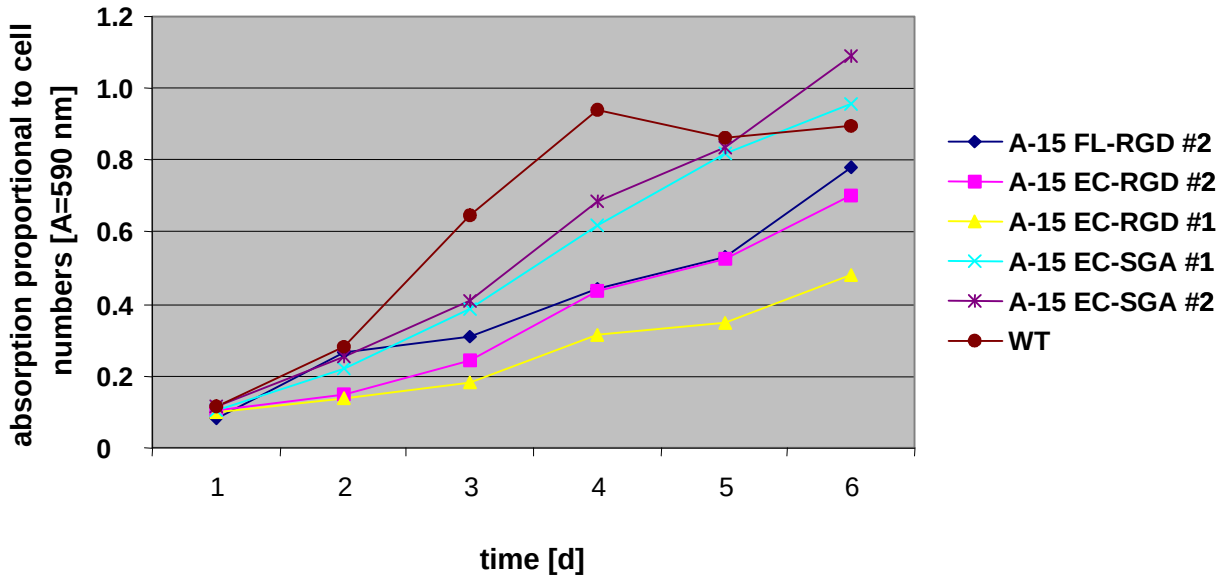


Figure 21 Evaluation of numbers of OV-MZ-6 cells adherent to VN as a function of ADAM15 overexpression by MTT assays over a period of 6 d
5,000 cells were plated on VN-pre-treated cell culture dishes. WT cell clones were used as a control After rinsing of the cell culture wells to wash away non-adherent cells, numbers of adherent cell were measured as described previously. Numbers of adherent A-15 EC-RGD #1 and #2 expressing cell clones were markedly reduced when compared to WT and A-15 EC-SGA mutant control cell clones. A-15 FL-RGD #2 cell clone numbers were significantly reduced when compared with WT and A-15 EC-SGA #1 and #2 mutant control cell clones. Depicted is a representative experiment.

After 6 d, cell numbers of the A-15 FL-RGD #2 cell clone cultivated on VN were reduced by 46 % when compared to WT cells, by 47 % when compared to the A-15 FL-SGA #1 control cell clones, and by 55 % when compared to the A-15 FL-SGA #2 control cells.

A representative MTT-test performed with ADAM15 EC overexpressing cell clones showed markedly reduced numbers of adherent A-15 EC-RGD-expressing cell clones when

compared to the A-15 EC-SGA mutant control cell clones and the WT cell clones. After 6 d, cell numbers of adherent A-15 EC-RGD cell clones compared to the A-15 EC-SGA #1 mutant cell clones were reduced by 50 % and 27 %, respectively. Numbers of adherent A-15 EC-RGD cell clones compared with the A-15 EC-SGA #2 mutant cell clones was decreased by 56 % and 35 %, respectively.

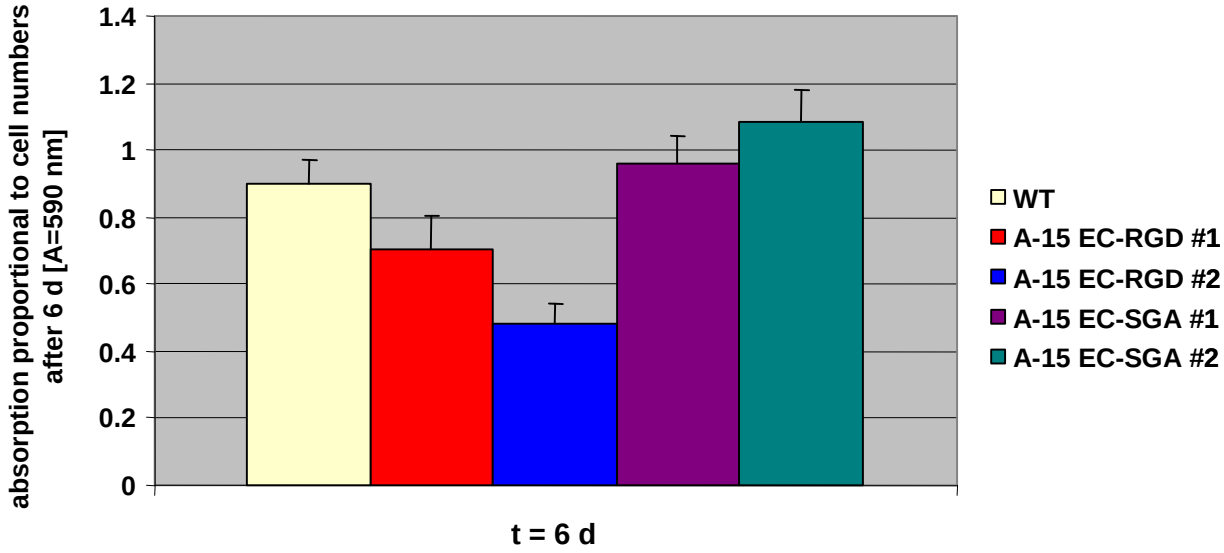


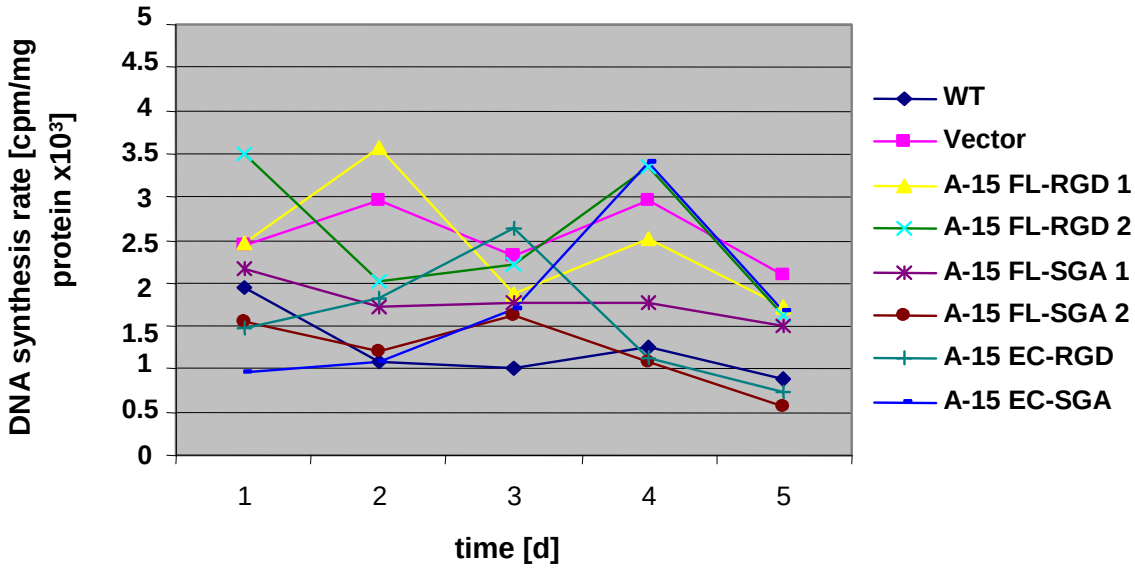
Figure 22 Evaluation of numbers of OV-MZ-6 cells adherent to VN as a function of elevated ADAM15 expression by MTT assays after 6 d
Comparison of absorption proportional to cell numbers after 6 d as depicted in fig 21. A marked reduction in adherent cell numbers can be found regarding the A-15 EC-RGD #1 and #2 cell clones when compared to the A-15-EC-SGA mutant control cell clones and the WT cell clones.

3.4.3 Measurement of *de novo* DNA synthesis in ADAM15-overexpressing OV-MZ-6 cells by [^{3}H]-thymidine incorporation assays

Human ovarian OV-MZ-6 cancer cells are of epithelial origin and strictly anchorage-dependent. In order to prove whether the observed decreases in cell numbers are due to cell loss by diminished cell adhesion upon ADAM15 overexpression in OV-MZ-6 cells or rather to altered cell proliferation rates, we determined the amount of *de novo* DNA synthesis of different ADAM15 overexpressing OV-MZ-6 cell clones by [^{3}H]-thymidine incorporation assays.

Hereby, it is possible to differentiate whether a change in cell numbers of ADAM15 overexpressing cell clones cultivated on VN is due to an increase or decrease in DNA synthesis rates, or rather due to an impact of ADAM15 on adhesion to the ECM.Every other 24 h, [^{3}H]-thymidine incorporation into cellular DNA was determined as described previously. In order to normalize incorporated radioactivity, protein concentrations of cells cultivated in parallel at the same density were determined as measure of cell numbers. Radioactivity incorporated into cellular DNA was expressed as counts per minute values. The [^{3}H]-thymidine incorporation of A 15 FL-RGD #1 and #2 cell clones shows that their levels of uptake remain fairly constant on both non pre-treated and VN-coated surfaces over a period of 5 d. Preliminary results on both surfaces hint to the fact that there are no significant alterations of *de novo* DNA synthesis induced by (over)expression of ADAM15.

A



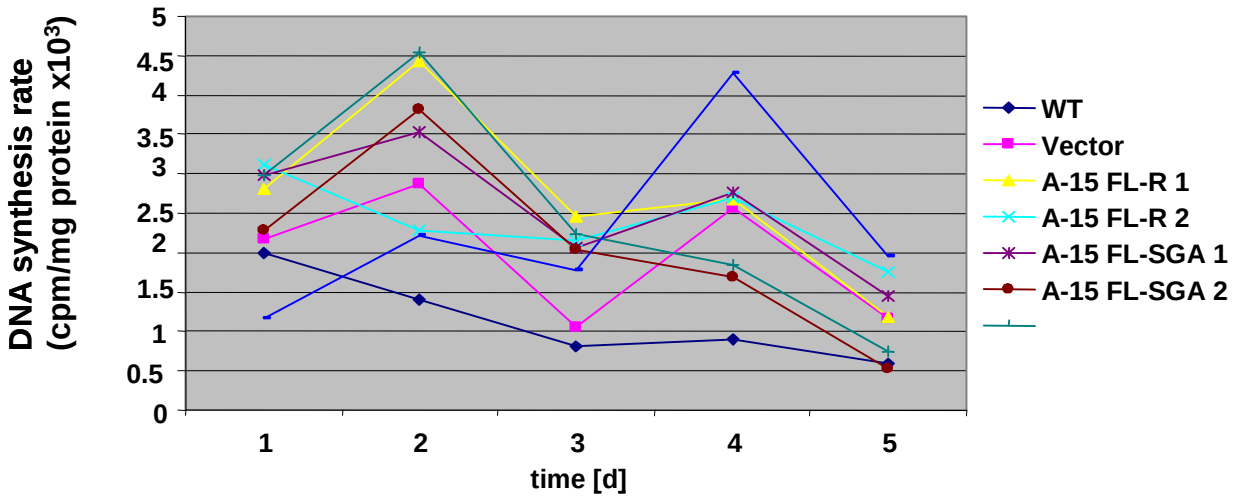
B

Standard deviation (SD)	Day 1	Day 2	Day 3	Day 4	Day 5
WT	14.7	23.8	4.3	16.2	27.3
Vector	54.4	27.7	24.5	35.2	13.2
A-15 FL-RGD 1	49.9	12.9	29.8	47	29,8
A-15 FL-RGD 2	42.6	48.9	38.9	26.3	12,3
A-15 FL-SGA 1	32.0	45.6	67.3	35,3	26,2
A-15 FL-SGA 2	27.7	42.7	19.3	39,2	21,3
A-15 EC-RGD	30.9	39,8	10.2	35,1	37,2
A-15 EC-SGA	31.3	24.9	18.3	48,2	11.2

Figure 23 A/B [^{3}H]-thymidine incorporation assay of ADAM15 overexpressing OV-MZ-6 cells cultivated on VN-coated cell culture plates
[^{3}H]-thymidine incorporation assay was performed as described. The A-15 FL-RGD and A-15 EC-RGD overexpressing cell clones do not show any significantly induced *de novo* DNA synthesis rate when compared to the A-15 FL-SGA control cells and the WT cells. Depicted is one typical experiment of three done in duplicates. Cpm values were normalized to the protein concentration of each cell sample harvested in parallel. Depicted in figure B are the standard deviations (SD) of each cell clone over the time period measured.

Incorporation levels of added [^{3}H]-methyl-thymidine into cellular DNA on VN were not higher than on the non pre-treated surface. This is not quite consistent with the fact that VN is known to induce proliferation in a variety of cells. As these results are merely preliminary further investigations will have to be conducted to verify this in the above setting. Despite fluctuations in the uptake of [^{3}H]-labeled thymidine throughout the period of 5 d, the *preliminary* results suggest that there is neither significant induction nor inhibition of *de novo* DNA synthesis caused by ADAM15 overexpression when compared to WT, A-15 FL-SGA, and A-15 EC SGA cell clones. This indicates that the observed differences in cell numbers are due to a loss of cells by reduced adhesion or detachment of A-15 RGD overexpressing cells rather than differences in *de novo* DNA synthesis.

A



B

Standard deviation (SD)	Day 1	Day 2	Day 3	Day 4	Day 5
WT	34.8	26.9	17.9	18.2	9,1
Vector	60.1	31.3	15.1	16.9	17,6
A-15-FL-RGD 1	25.9	40.4	11.1	35.2	24.5
A-15 FL-RGD 2	57.3	64.7	15.2	28.2	24.7
A-15 FL-SGA 1	79.7	139.4	4,2	3.2	31.2
A-15 FL-SGA 2	12.0	112.5	9,2	7.3	37.2
A-15 EC-RGD	32.6	38.9	10,2	35.2	34.2
A-15 EC-SGA	28.2	55.9	12,2	41.4	26.5

Figure 24 A/B [^{3}H]-thymidine incorporation assay of ADAM15 overexpressing OV-MZ-6 cells cultivated on non-pretreated cell culture plates

The incorporation of [^{3}H]-thymidine of all cell clones used in the experiment was lower on the non pre-treated surface than on the VN-coated surface. The *de novo* DNA synthesis of the A-15 FL-RGD and A-15 EC-RGD overexpressing cell clones per cell was not significantly altered when compared to the WT cells and the A-15 FL-SGA mutant control cells. Depicted is one typical experiment of three done in duplicates. Cpm values were normalized to the protein concentration of each cell sample harvested in parallel. Figure B depicts standard deviations of all cell clones over the time period measured.

4. Discussion

Ovarian cancer is one of the most aggressive gynecological cancers showing its symptoms late, and is consequently often diagnosed at an advanced stage. The disease is known to spread early resulting in a considerable number of patients dying due to metastases. In fact, it is the leading cause of death from gynecological malignancies in most western countries. Treatment so far consists of cytoreductive surgery ("debulking"), taxol platinum-based chemotherapy, and irradiation. Mortality rates are still not very satisfying, however.
This encourages the search for new methods of earlier diagnosis and more sophisticated therapies. New approaches, such as immunotherapy or gene therapy could possibly become important treatment options in the future, though they have not reached clinical significance yet. Thus, the search for new approaches to the treatment and prevention of ovarian cancer is pursued intensely.

In general, tumor proliferation, invasion and, eventually, metastasis are the underlying reasons of most tumor deaths. Consequently, new therapeutic strategies have to be directed towards this cascade in order to have the desired impact on survival rates (Woodhouse et al., 1997, 1529-37).
Tumor cells are dependent on a variety of factors ensuring their survival and potential to proliferate and metastasize. For example, tumor growth factors and changes in the surrounding ECM facilitate the progression of the malignant state (Liotta et al., 2001, 375-9). Furthermore, neovascularization to further nourish the tumor is an essential part of tumor survival strategies (Cristofanilli et al., 2002, 415-26). Adhesive receptors like cadherins and integrins play a vital part in tumor survival and proliferation and are able to mediate (neo-)angiogenic events. They also mediate adhesion and detachment from the ECM and transmit signals into and out of the cells. The subsequent signaling cascade is capable of facilitating tumor progression (Giancotti et al., 1999, 1028-32, Guo et al., 2004, 816-26).
Regarding tumor cell adhesion and detachment from the ECM – vital prerequisites for tumor migration and, eventually metastasis – particular importance can be attributed to the adhesion and signaling receptors of the superfamily of the integrins. In the case of ovarian cancers, which predominantly spread in the peritoneal cavity integrins could mediate tumor cell dissemination in the intraperitoneal cavity and adhesion to organs and the peritoneum. In particular, integrin $\alpha v\beta 3$ is involved in angiogenesis (Brooks et al., 1995, 1815-22; Sipkins et al., 1998, 623-6; Chen at al., 2004, 44166-76), tumor

invasion and metastasis (Seftor et al., 1999, 359-75; Voura et al., 2001, 2699-710; Pilch et al., 2002, 21930-8) and has evolved into a prime target for anti-cancer drugs (Jin et al., 2004,

561-5). Integrin αvβ3 has been found on malignant cells, such as melanoma cells (Marshall et al., 1991, 924-31), mamma carcinoma cells (Gasparini et al., 1998, 2625-34), and ovarian cancer cells (Cannistra et al., 1995, 216-25). Ovarian cancer cells of a higher malignant potential express integrin αvβ3 at a higher level than ovarian cancer cells with a lower malignant potential (Liapis et al., 1997, 443-9). Ovarian surface epithelium cells synthesize integrin αvβ3 and its natural ligand, VN (Carreiras et al., 1996, 260-7, Cruet et al., 1999, 254-60). VN is also synthesized by ovarian cancer cells and is one of the primary adhesion substrates within this context. Through its RGD motif, VN interacts with integrin αvβ3. Activation of integrin αvβ3 is associated with enhanced cell invasiveness in a variety of tumors, such as human melanomas (Ria et al., 2002, 836-45), enhanced cancer progression in prostate cancer, and breast cancer (Felding-Habermann et al., 2001, 1853-8, Cooper et al., 2002, 191-4), and reduced survival rates in colon carcinomas (Vonlaufen et al., 2001, 1126-32).

Ovarian cancer cells, such as OV-MZ-6 cells have been shown to organize their adhesion to VN predominantly through the activated integrin αvβ3 in an RGD-dependent way. Moreover, VN as underlying matrix significantly induces cell adhesion, proliferation, and migration in OV-MZ-6 cells in an αvβ3-dependent fashion (Hapke et al., 2003, 1073-83). Interaction of integrin αvβ3 with VN has been found to induce and facilitate cell proliferation, adhesion, and invasion of a variety of malignant cells, such as human ovarian cancer cells, T-cell lymphoblastic leukemia cells (Vacca et al., 2001, 993-1003), and multiple myeloma cells (Ria et al., 2002, 836-45). Considering the importance of integrin αvβ3 in cancer settings and its interaction with VN, this interaction has evolved into a potential target for cancer treatment (Jin et al., 2004, 561-5).

This seems also important from an evolutionary point of view as combinatorial antibody libraries contain the entire immunoglobulin pool ever synthesized by the respective individual. Upon generation of this “fossil record” of an individual’s immune response in individuals with a history of breast cancer, it yielded human antibodies specific for the activated conformation of integrin αvβ3. Two out of these antibodies contained the RGD motif underlining the potential evolutionary importance of this integrin αvβ3-mediated interaction in a cancer setting (Felding-Habermann et al., 2004, 17210-5).

Synthetic RGD peptides and antibodies directed to integrins that are thought to competitively interact with integrin αvβ3-mediated binding to the ECM have been explored *in vitro* and *in vivo* regarding their efficacy. *Vitaxin* is a humanized monoclonal IgG1 antibody that specifically binds a conformational epitope formed by both the integrin alphaV and beta3

subunits. It blocks the interaction of αvβ3 with various ligands such as osteopontin and vitronectin (Wilder, 2002, 96-9). C*ilengitide* is a cyclic peptide mimicking the RGD ligand recognition peptidic domain common to alpha v integrin ligands. Both *Vitaxin* (αvβ3-antibody) and C*ilengitide* (RGD-peptide) are in phase II clinical trials (Tucker, 2006, 96-103). So far, these peptides and antibodies have shown promising results. They have been shown to promote tumor regression and reduce angiogenesis (Brooks et al., 1994, 1157-64; Dechantsreiter et Kessler, 1999, 3033-40; Kok et al., 2002, 128-35, Shannon et al., 2004, 129-38, Haubner et al., 2004, 1439-55).

Besides synthetic integrin antagonists, another group of naturally occurring integrin antagonists/ligands has been discovered in recent years. These are part of the ADAM family of proteins. Within this family, human ADAM15 stands out, for it is the only ADAM to contain an RGD-motif in its disintegrin domain. The disintegrin domain of ADAM15 has been shown to specifically interact with integrin αvβ3 in an RGD-dependent way (Zhang et al., 1998, 7345-50; Nath et al., 1999, 579-87).

4.1 ADAM15 overexpressing human ovarian cancer cells show decreased adhesive capacitiy towards vitronectin

Human ADAM15, featuring an RGD motif in its disintegrin domain could compete for the interaction between integrin αvβ3 and VN. Due to the endogenous expression of ADAM15 in human OV-MZ-6 ovarian cancer cells, ADAM15 could therefore function as a natural integrin antagonist for this important adhesive cell interaction.

In order to investigate the effect of ADAM15 *over*expression on the adhesive behavior of human ovarian cancer cells and ADAM15 expressing CHO cells, these cells were stably transfected with both FL and EC ADAM15 cDNAs. ADAM15 FL and ADAM15 EC *over*expressing OV-MZ-6 cell clones and ADAM15 expressing CHO cell clones showed a significantly reduced adhesion to VN in an RGD-dependent fashion, whereas expression of ADAM15 molecules displaying an SGA instead of an RGD motif, as well as WT or vector-transfected cell clones did not show a significant reduction of cell adhesion. The reduction of cell adhesion could possibly be due to a direct, competitive interaction of the RGD motif of ADAM15 with integrin αvβ3. Hereby, the natural ECM ligand of integrin αvβ3, the RGD motif displayed by VN, would be competitively antagonized by human ADAM15. In principle, this interaction could occur on the very same cell with the ADAM15 RGD motif binding to αvβ3 expressed on the same cell in direct local proximity (*intra*cellular ADAM15-αvβ3 antagonism). It is also conceivable that ADAM15 via its RGD motif could interact with integrins on adjacent cells (*inter*cellular ADAM15-αvβ3 antagonism). This could lead to an increase in cell-cell contacts and a decrease in cellular migration. This was also shown by Charriet and coworkers (2005) who demonstrated a reduced cell migration in wound healing assays using human intestinal epithelial cells. Herren and coworkers (2001) also demonstrated a decrease in cell migration upon ADAM15 overexpression. They showed that elevated ADAM15 inhibits migration of NIH3T3 cells on FN. This phenomenon was not due to altered ECM attachment or to lack of extracellular signal-regulated kinase (ERK) signaling response to FN, but rather depended on increased cell/cell interaction. This is particularly interesting as ADAM15 was detected in cellular adherens junctions where it is co-localized with vascular endothelial cadherin (Ham et al., 2002, 239-47). Both modes of interaction would impair or prevent interaction of integrin αvβ3 with its recognition motif, RGD, within its natural ECM ligand VN. Hereby, cells would be more easily detached from the surrounding ECM and become less adhesive. This working hypothesis was confirmed by the results of our adhesion assay experiments where ADAM15 FL and EC expressing human ovarian cancer cell clones exhibited significantly reduced adhesion on VN-coated cell growth surfaces when compared to WT cells, vector-transfected cells, or cells displaying an SGA

instead of an RGD motif. The competitive interaction of the disintegrin domain of ADAM15 with integrin αvβ3 could also facilitate apoptosis of the affected (tumor-)cells, as VN has been found to protect tumor cells from apoptosis (Uhm et al., 1999, 1587-94). Competitive ADAM15 interaction with integrin αvβ3 *in vivo* may as well block neointima formation by smooth muscle cells, since this process is partially mediated by VN-integrin αvβ3 interaction (Dufourcq et al., 2002, 952-62). Neointima formation, however, is mediated by a number of other integrin-VN interactions so that a broader array of inhibiting antibodies would be necessary for complete blockage.

Since human ADAM15 also contains a (potentially) proteolytically active MP domain (Martin et al., 2002, 33683-9), it is possible that this domain is used to direct the disintegrin domain of ADAM15 to a corresponding integrin as a form of "targeted migration". This bifunctional potential of human ADAM15 makes it particularly interesting when compared to more monofunctional molecules like, i.e. MMPs. In fact, preliminary but not yet confirmed results point to ADAM15 being an active MP degrading both Col IV - an important constituent of basement membranes - and gelatin (Martin et al., 2002, 33683-9). Basement membranes constitute a crucial part in the cascade of tumor metastasis, since the basement membrane can be the last barrier before dissemination of cancerous cells into the peritoneal cavity in the case of ovarian cancer. As this is the only report so far suggesting an active MP domain, these results need further confirmation by, e.g. examining cells genetically deficient in ADAM15. Maximum cell migration in the ECM is expected to occur at intermediate cell-substratum adhesiveness. ADAM15 expression could potentially facilitate migration of cells within the ECM by loosening cell substratum contacts and forming part of carefully controlled system of adhesion, detachment and migration that is highly significant in cancer settings (Herren et al., 2001, 152-60).

Indeed, ADAM15 expression is often elevated in a number of conditions characterized by extensive ECM remodeling, like, i.e. during inflammation, in atherosclerotic lesions, or in malignancies (Carl-McGrath et al., 2005, 17-24; Lendeckel et al., 2005, 41-8). In these settings, it would be conceivable that ADAM15 expressing cells or the shedded EC domain of ADAM15 could be directed towards the location of ECM degradation and take part in tissue remodeling through their MP domain. This migration would be supported by the ADAM15 disintegrin domain, which constitutes part of a fine-tuned balance between the formation and loosening of adhesive cell contacts that is essential in tissue remodeling (Hapke et al., 2003, 1073-83).

Unlike MMPs that are known to be involved in ECM degradation and lack a disintegrin domain to direct them towards their location of activity, ADAM15 expressing cells combine both distinct domains and potential functions.

However, there is contradictory evidence of ADAM15 overexpressing cells` migratory behavior, which can be partially considered a result of their adhesive phenotype. Herren and coworkers (2001) demonstrated that ADAM15 overexpressing NIH3T3 cells showed a decreased migration due to their increased cell-cell interaction *in vitro*. This would be compatible with the theory that ADAM15 overexpressing cells competitively interact with integrins, such as αvβ3 or α9β1 on adjacent cells and decrease migration through these increased cell-cell contacts. ADAM15 may mediate cell-cell interaction in metastatic settings leading to homotypic cell aggregation. This is in accordance with recent findings showing that ADAM15-deficient mice show significantly reduced neovascularization and tumor growth (Horiuchi et al., 2003, 5614-24). Contrary to what we expected, however, my coworkers have not found any effect of ADAM15 overexpression on the extent of cell-cell interaction in cell-cell adhesion assays (data not shown). However, the αvβ3-antagonistic effect of cyclic RGD-peptides is dependent on the antagonist concentration. Low concentrations of the ligand mimetic cyclo-RGD can result in superactivation of the extracellular domain of integrin αvβ3 to a comparable level as activation by manganese. (Legler et al., 2001, 1545-53). It is conceivable that in the above setting a higher antagonist concentration - equivalent to ADAM15 expression on the OV-MZ-6 cells - is necessary to affect αvβ3-mediated cell-cell interaction. Probably, ADAM15 expression is both necessary for migration of cells within the ECM and capable of inhibiting migration by facilitating cell-cell contacts. It remains unclear, however, if ADAM15 has an effect on cell migration and cell-cell interaction when expressed at endogenous levels in non-transfected cells as these levels might not be sufficient for adequate antagonism, leading to superactivation of integrin αvβ3 instead (Legler at al., 2001, 1545-53). This could also depend on cytokine signaling, and the impact of (tumor) growth factors.

Decreased adhesion of ADAM15 overexpressing human ovarian cancer cells to the ECM could be part of a larger fine-tuning system involved in remodeling the microenvironment of tumors, atherosclerotic lesions or atrial dilatation. The unique ability of ADAM15 to both bind to integrins and, potentially, degrade parts of the ECM remains to be further investigated. Hereby, many other factors are likely to contribute, amongst these other members of the ADAM family of proteins, MMPs, and the plasmin/urokinase-plasminogen activator system.

4.2 Time-dependent decline in cell numbers of ADAM15 overexpressing ovarian cancer cells attached to vitronectin is a result of diminished adhesive strength rather than altered cell proliferative activity

Adhesion to the ECM is a prerequisite for proliferation of anchorage-dependent, epithelium-derived tumor cells, including ovarian cancer cells. Hereby, cell-cycle progression is in part controlled by integrin-mediated adhesion to the ECM and by consecutive binding of growth factors to their corresponding receptors. Mediation of these processes ensues from G1 phase cyclin-dependent-kinases (CDKs). These CDKs can be found downstream of signaling pathways and are under the control of integrins, such as $\alpha v\beta 3$ and growth factor receptors. Recently, a wide array of integrin-mediated signals, which lead to the regulation of G1 CDKs, has been discovered. Thus, the connection between cell adhesion and cell proliferation of adherent cells comprises a closely-knit network of integrins, growth factors, signaling-cascades and CDKs (Schwartz et al, 2001, 2553-60).

In order to investigate the effect of ADAM15 overexpression on changes in cell numbers of human ovarian cancer cells over time, we conducted cell counting and MTT assays. In order to be able to differentiate between a decrease in cell numbers of ADAM15 overexpressing ovarian cancer cells over time due to a reduced *de novo* DNA synthesis or a reduced cell adhesion, in parallel we conducted [^{3}H]-thymidine incorporation assays.

Numbers of adherent ADAM15-overexpressing human ovarian cancer cells were significantly decreased over time as a function of the presence of an RGD motif within the ADAM15 disintegrin domain. Compared to the ADAM15 control cell clones displaying an SGA motif in their disintegrin domain, both the ADAM15 FL- and the ADAM15 EC-(over)expressing cell clones showed decreased cell numbers when seeded on the purified ECM protein VN. ADAM15 (over)expressing cells at the same time showed - as shown in the adhesion experiments - a significantly reduced adhesion to VN, possibly due to an antagonistic effect on VN/$\alpha v\beta 3$ interaction. This was confirmed by the results of the [^{3}H]-thymidine incorporation assays. Hereby, we tried to differentiate if a reduction of cell numbers over time was due to altered patterns of *de novo* DNA synthesis or rather due to a change in adhesion due to ADAM15-overexpression. We did not see any significant alterations in *de novo* DNA synthesis per cell over time. Thus, the cell loss over time of ADAM15 overexpressing ovarian cancer cells in the [^{3}H]-thymidine incorporation assays seems to be due to non re-adherence of the anchorage-dependent tumor cells rather than changes in *de novo* DNA synthesis. This indicates that the phenomenon of reduced cell numbers could be due to decreased cell adhesion caused by competitive interference of the

RGD-motif of ADAM15 with integrin αvβ3 leading to a reduced interaction of integrin αvβ3 with its natural ECM ligand VN. This interference could possibly also have an effect on the further downstream signaling-cascade (Schneller et al., 1997, 5600-7; Soldi et al., 1999, 882-92).
Overall, highly complex settings, such as atherosclerotic lesions or tumors are vulnerable to numerous factors, which could have an influence on the actual rate of neovascularization or metastasis.

4.3 Summary

Human ADAM15 is a member of the ADAM family of proteins. It has been found in numerous physiological and pathophysiological settings, and it is unique since it is the only ADAM known so far to contain an RGD motif in its disintegrin domain. The RGD motif could possibly interact with integrins, such as integrin αvβ3. The interaction between the ADAM15 RGD motif and the integrin αvβ3 could occur on the same cell or with an integrin on adjacent cells. Either way, ADAM15 could function as a naturally occurring integrin antagonist. The result of both interactions would be an interference of ADAM15 with the natural ligand of integrin αvβ3, e.g. vitronectin. The natural ligand of integrin αvβ3 in the ECM is the glycoprotein VN. Synthetic integrin αvβ3 antagonists, such as cyclic RGD-peptides have already been developed in order to tackle the interaction between integrin αvβ3 and its ligands. This is based on the knowledge that integrin αvβ3 is an important factor in tumor growth and metastasis.
In our experiments, ovarian cancer cells, OV-MZ-6, and CHO cells were stably transfected with ADAM15 cDNA. This resulted in overexpression of ADAM15 in the (endogenously ADAM15 expressing) OV-MZ-6- cells and expression of ADAM15 in the CHO cells, as confirmed in RT-PCRs and CLSM imaging. In adhesion assays on VN, the ADAM15 (over-) expressing cells showed a significantly decreased adhesion in an RGD-dependent manner when compared to the ADAM15 SGA mutant control cell clones and WT and vector-transfected control cell clones. The decrease in adhesion was significant on VN where ADAM15 potentially interferes with the interaction of the integrin αvβ3 and its natural ligand VN. As a result of this interference, cellular adhesion of, i.e. atherosclerotic and cancerous cells to their ECM could be loosened and cells could detach more easily. This may lead to an increased cell-cell interaction and, consequently, reduced cell migration. This is conceivable as ADAM15 was detected in cellular adherens junctions where it colocalizes with vascular endothelial cadherin. As a result, ADAM15 may foster neovascularization and

tumor growth which is supported by the fact that ADAM15 –deficient mice show significantly reduced neovascularization and tumor growth.

In our experiments evaluating cell numbers over time, e.g. by cell-counting assays or MTT-assays, cell numbers of the ADAM15 overexpressing ovarian cancer cells were significantly reduced when compared to the control cells. This reduction is probably due to the impaired adhesion, as OV-MZ-6 cells are anchorage-dependent in order to proliferate. Also, the disrupted adhesion between integrin $\alpha v\beta 3$ on the ovarian cancer cells and the ECM protein VN could potentially interfere with the downstream cytoplasmic signaling cascade. ADAM15 could also directly interact with Src family proteins via its cytoplasmic domain, as has been show for its two splicing variants ADAM 15v1/v2 in lymphocytes. Hereby, ADAM15 could influence immunological and inflammatory processes like, i.e. atherosclerosis and osteoarthritis.

Overall, ADAM15, as has been demonstrated in our experiments is an important cell surface protein capable of interfering with adhesion between integrin $\alpha v\beta 3$ and its natural ECM ligand, VN. This makes ADAM15 a naturally occurring integrin antagonist. Considering the crucial importance of integrins and the many processes they are involved in, ADAM15 will certainly be of high interest for future research

Future experiments could, i.e. be directed at determining a potential correlation of ADAM15 expression in primary ovarian cancer and peritoneal metastases *in vivo*.

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