Forschungszentrum Karlsruhe
Technik und Umwelt

Wissenschaftliche Berichte
FZKA 6714

Conditional Expression of CD44 Isoforms in Lymphoma Cells: Influence on Hyaluronate Binding and Tumor Growth

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der Universität Karlsruhe (TH)
genehmigte Dissertation

Forschungszentrum Karlsruhe GmbH, Karlsruhe
2002

Impressum der Print-Ausgabe:

Als Manuskript gedruckt

Forschungszentrum Karlsruhe GmbH
Postfach 3640, 76021 Karlsruhe

Mitglied der Hermann von Helmholtz-Gemeinschaft
Deutscher Forschungszentren (HGF)

ISSN 0947-8620

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Zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

an der Fakultät für Bio-und Geowissenschaften

der Universität Karlsruhe

genehmigte

DISSERTATION

von

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aus Yunhe, China

2002

Tag der mündlichen Prüfung: 30.01.2002
Referent: Prof. Dr. Helmut Ponta
Korreferent: Prof. Dr. Margot Zöller

Konditionale Expression von CD44 Isoformen in Lymphomzellen: Einfluß auf Hyaluronsäurebindung und Tumorwachstum

Zusammenfassung

CD44 bezeichnet eine Familie von Zellmembranproteinen, die sich aus verschiedenen Isoformen zusammensetzt, die durch alternatives Spleissen von zehn „varianten“ Exons generiert werden. Mitglieder dieser Familie sind an unterschiedlichen Prozessen beteiligt, wie zum Beispiel an Hämatopoiese, Aktivierung und Homing von Lymphozyten, Gliedmaßenentwicklung, Wundheilung und Tumorprogression. Es wurde gezeigt, daß CD44 ein prognostischer Parameter für verschiedene Krebserkrankungen des Menschen ist.

Um die Frage zu beantworten, ob bestimmte CD44 Isoformen für die Tumorprogression relevant sind und um Einsicht in den Mechanismus ihrer Funktion zu erhalten, habe ich Transfektanten der LB Lymphomzelllinie etabliert, in denen die Expression von vier CD44 Isoformen, nämlich CD44v3-10, CD44v4-10, CD44v8-10 und CD44s, unter der Kontrolle des Tet-off Promotors steht. In der Anwesenheit von Doxyzyklin war die Expression reprimiert. Entfernung von Doxyzyklin schaltete die Expression ein und ein maximalen CD44 Proteingehalt wird nach zwei Tagen erhalten. Die Transfektanten wurden bezüglich ihrer Fähigkeit zur Bindung von Hyaluronsäure (HA) charakterisiert, einer Komponente der extrazellulären Matrix. Überexpression aller vier CD44 Isoformen bewirkte die Bindung von HA an LB Zellen. Andere Glykosaminoglykane (GAG) wurden in isotypspezifischer Weise gebunden. CD44v3-10, CD44v4-10 und CD44v8-10 zeigten eine hohe Bindungsaffinität für Chondroitin A, B und C, und eine niedrige Affinität zu Heparin, Heparansulfat und Keratansulfat. CD44s konnte an diese GAGs nicht binden. Unter den drei Variantenisoformen war die Bindungsfähigkeit von CD44v3-10 zu den GAGs am größten.

CD44 „Clustering“ scheint eine kritische Bedeutung für die Bindung von HA zu haben. Sowohl CD44s als auch CD44v8-10 bildeten reduktions-sensitive Komplexe in LB Zellen. Die Komplexe bestehen aus Homo- oder Heterooligomeren verschiedener CD44 Isoformen. Cys286 in der Transmembran-Domäne von CD44 war für die Bildung reduktions-sensitiver Oligomere und für die vermehrte HA-Bindung in LB Zellen nicht verantwortlich. In einem konditionalen

Dimerisierungssystem wurde die Notwendigkeit der CD44 Oligomerisierung für die Bindung von HA direkt gezeigt. Die Induktion der Oligomerisierung verstärkte die Bindung von HA.

Abschließend untersuchte ich die Rolle von CD44 in der Entwicklung von Lymphomen durch subkutane Injektion der Transfektanten in syngene oder immundefiziente Mäuse. Ich fand keinen Einfluß von CD44v3-10, CD44v4-10 und CD44v8-10 Isoformen auf das Wachstum von Lymphomen und ihrer Metastasierungsfähigkeit.

Abstract

CD44 describes a family of surface proteins consisting of many isoforms due to alternative splice of ten ″variant″ exons. Members of this family are involved in various processes including hematopoiesis, lymphocyte activation and homing, limb development, wound healing and tumor progression. Clinically, CD44 has been shown to be a prognostic factor for several human cancers.

To answer the question which isoform might be relevant for tumor progression and to gain an insight into the mechanism of its function, I established transfectants of the LB lymphoma cell line in which the expression of four CD44 isoforms, namely CD44v3-10, CD44v4-10, CD44v8-10 and CD44s, was controled by the Tet-off promoter. In the prescence of Doxycycline, the expression was repressed. Removal of Doxycycline switched on expression and the maximal CD44 amount was obtained within two days.

The transfectants were characterized regarding their ability to bind to the extracellular matrix component hyaluronate (HA). Overexpression of all four CD44 isoforms conferred the ability to bind HA on LB cells. Other glycosaminoglycans (GAGs) were bound in an isotype-specific fashion. CD44v3-10, CD44v4-10 and CD44v8-10 showed high binding affinity to chondroitin A, B and C, and low affinity to heparin, heparan sulfate and keratan sulfate. CD44s could not bind to these GAGs. Among these three variants, the binding ability of CD44v3-10 was the strongest.

CD44 clustering seemed to play a crucial role for HA binding. Both CD44s and CD44v8-10 formed reduction-sensitive complexes in LB cells. The complexes are homooligomers or heterooligomers composed of different isoforms. Cys286 in CD44 transmember domain was not responsible for the formation of reduction-sensitive oligomer or for the enhanced HA binding in LB cell line. Using a conditional dimerization system the requirement of CD44 oligomerization for HA binding was directly demonstrated. The induction of oligomerization increased HA binding.

Finally, I studied the role of CD44 in lymphoma development by subcutaneous injection of the transfectants in syngeneic or immunocompromised mice. I found no influence of CD44v3-10, CD44v4-10 and CD44v8-10 isoforms on the LB lymphoma formation and metastasis.

Acknowledgements

I here thank Professor Dr. Peter Herrlich for providing me with the opportunity to pursue my PhD in Institute of Genetics, Forschungszentrum Karlsruhe. I benefited much from both his broad view of science and incessant financial support.

I take this chance to give my gratefulness to Professor Dr. Helmut Ponta for his contribution of ideas to my PhD work, especially for his huge input to instructing my thesis writing and to correcting my thesis.

I appreciate all the supervision and help from Dr. Martin Hegen during the first two years of my PhD.

I am very thankful to the contribution of ideas, discussion of data and helps in each type of form from Dr. Christoph Englert, Dr. Harald König and Dr. Jonathan Sleeman.

I thanks a lot for the precious help from: Michal, Natascha and Vladmir, Norma, Jaya, Helen, Susanne, Chinese cohorts (Yong Li, Ping, Chen…), Johnboy, Selma, Birgit, Berti, Dagmar, Ingrid, Marga, Uschi, Petra and all the people who ever helped me.

I also thank Professor Dr. Margot Zöller for reading and correcting this thesis for me.

Finally, I want to express my thankfulness to my family, especially my wife Yulan who accompied me in the past time, my mother-in-law, my daughter, and my parents for their generous support, mentally and physically, from which I can always achieve motivation.

INDEX

Abbreviations

A	alanine
aa	amino acid
Ab	antibody
AER	apical ectodermal ridge
Ala	alanine
Arg	arginine
Asn	asparagine
b-FGF	basic fibroblast growth factor
BS^3	Bis(sulfosuccinimidyl) Suberate
BSA	bovine serum albumin
Ca	calcium
$CaCl_2$	calcium chloride
CAM	cell adhesion molecule
CD44s	CD44 standard
CD44v	CD44 variant
CDK	cycline-dependent kinase
CIP	calf intestine phosphatase
cm	centimeter
CMV	cytomegalovirus
CO_2	carbon dioxide
CPC	Cetylpyridinium Cholate
CS	chondroitin sulfate
Cys	cysteine
ddH_2O	double-distilled water
ddNTPs	2´,3´-dideoxyribonucleosides triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
Dox	doxycycline
DTSSP	Dithiobis(sulfosuccinimidyl propionate)
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	ethylene diaminetetraacetic acid
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
EGTA	ethyleneglycol bis(2-aminoethylether) tetraacetic acid

ERK	extracellular signal-regulated kinase
ERM	ezrin, radixin, and moesin
Fab	fragment of antigen binding
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	Fluorescein Isothiocyanate
FKBP	FK506-binding protein
FRET	fluorescence resonance energy transfer
g	unit of relative centrifugal force
G418	Geneticin
GAG	glycosaminoglycan
GEF	guanine nucleotide exchange factor
GF	growth factor
GFP	green fluorescent protein
Gly	glycine
GTPase	guanosine triphosphatase
H	heparin
HA	hyaluronate
Has	HA synthases
HB-EGF	heparin-binding epithelial growth factor
HGF/SF	hepatocyte growth factor/scatter factor
HPSF	High Purity Salt Free
hr	hour
HRP	horseradish peroxidase
HS	heparan sulfate
ICAM	intracellular cell adhesion molecule
Ig	immunoglobulin
K	lysine
kD	kilo dalton
KS	keratan sulfate
LEF-1	leukocyte enhancer factor-1
Leu	leucine
LFA	lymphocyte function-associated antigen
M	molar per litre
mAb	monoclonal antibody
β-ME	β-mercaptoethanol
MFI	mean fluorescence intensity
mg	milligram
Mg	magnesium

$MgSO_4$	magnesium sulfate
min	minute
ml	millilitre
mM	milli molar per litre
MMP	matrix metalloproteinase
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	messenge ribonucleic acid
MW	molecular weight
NaCl	sodium chloride
NaN_3	sodium azide
NaOH	sodium hydroxide
ng	nanogram
NHS-LC-Biotin	Sulfosuccinimidyl-6-(biotinamido)hexanoate
PAGE	polyacrylamide gel electrophoresis
PAI	inhibitor of plasminogen activator
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	R-Phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
R	arginine
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	round per minute
RT	reverse transcription
SDS	sodium dodecyl sulfate
sec	second
ser	serine
SGSG	Ser-Gly-Ser-Gly
SSV	simian sarcoma virus
SV40	simian virus 40
TAE	Tris/acetate/EDTA electrophoresis buffer
Tc	tetracycline
TetR	tet repressor
Tiam 1	T lymphoma invasion and metastasis 1
TGF	transforming growth factor
TIMP	tissue-specific inhibitor of matrix metalloproteinase
tPA	tissue plasminogen activator

TRE	tet responsive element
tTA	tetracycline-controlled transactivator
Tyr	tyrosine
µg	microgram
µl	microlitre
µM	micro molar per litre
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
UV	ultraviolet
v	variant
VEGF	vascular endothelial growth factor
VHL gene	von Hippel-Lidau gene
VP16	virion protein 16
w/o	without
WT	wild type

PART ONE

INTRODUCTION

CD44 is the name of a family of type I transmembrane glycoproteins that are wildly expressed on a varity of cell types including most hematopoietic cells, epithelial cells and some endothelial and neural cells. CD44 proteins are engaged in various physiological and pathological processes such as hematopoiesis, lymphocyte activation and homing, limb development, wound healing, delayed type hypersensitivity and tumor progression (Herrlich et al., 1998; Naor et al., 1997).

1.1 The structure of CD44

All CD44 family members are encoded by a single gene that consists of 20 exons (Screaton et al., 1993; Screaton et al., 1992). The first 5 exons (exons 1-5) are present in all CD44 isoforms (constant exons), whereas the following 10 exons are subjected to alternative splicing (so-called variant exons, v1-v10) (Fig. 1). Among the last 5 exons (exons 15-19), exons 15, 16 and 17 are expressed in all CD44 isoforms, while exons 18 and 19 are also alternatively spliced (Screaton et al., 1992). Differential utilization of exons 18 and 19 generates a short version (3 amino acids (aa)) (Goldstein et al., 1989) and a long version (70 aa) of the cytoplasmic tail, among which the small version is expressed only in tiny amounts. (Stamenkovic et al., 1989). The most abundant CD44 isoform is standard CD44 (CD44s) which contains constant exon 1-5, 15, 16, 17, and 19, whereas CD44 variant isoforms resulting from alternative splicing of the 10 variant exons occur rather rarely (Günthert et al., 1991; Tölg et al., 1993). Post-translational modifications with N- and O-linked glycosylation and glycosaminoglycan (GAG) side chains further add to the diversity of CD44 (Jackson et al., 1995; Jalkanen and Jalkanen, 1992; Stamenkovic et al., 1989).

CD44s is made up of 270 aa comprising the extracellular domain, 23 aa defining the transmembrane domain and 70 aa establishing cytoplasmic domain (Lesley and Hyman, 1998; Screaton et al., 1992; Stamenkovic et al., 1989). 180 aa at the amino

terminal end are conserved among mammalian species. They contain six cysteine residues which can form three disulfide bonds and five conserved N-glycosylation

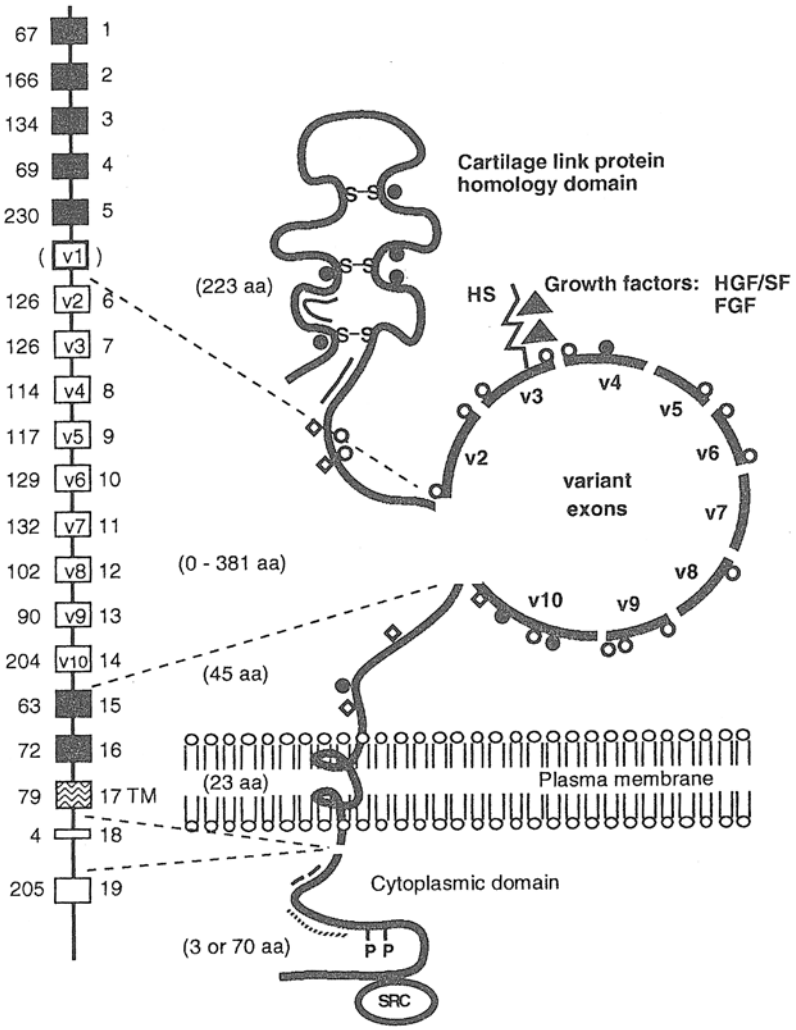


Figure 1. The schematic representation of CD44 gene and its encoded proteins. The extracellular domain and cytoplasmic tail of CD44 isoforms vary in size as the result of alternative splicing. The alternatively spliced exons are indicated by open boxes. The human v1 exon contains a stop codon. In the model of protein, all putative glycosylation sites are indicated: O-glycosylation (open circles); N-glycosylation (closed circles); chondroitin sulfate (open squares); heparan sulfate chain (rod). As indicated, the heparan sulfate binding site in exon v3 has the ability to bind growth factors. In addition, the HA-binding sites (double lines); the disulfide bonds (S-S) bonds; the ankyrin binding site (…); the ezrin binding sites (---); the phosphorylation sites (p); and the putative interaction sites for SRC family kinases, are indicated.

consensus sequences (at Asn 25, 57, 100, 110 and 120). Included in this amino terminal region is a ~100 aa link module which is important for the binding of CD44 to its principal ligand, hyaluronate (HA). The less conserved region (aa 183-256) includes potential sites for the addition of O-linked glycosylation, keratin sulfate and chondroitin sulfate (Takahashi et al., 1996). The sequence of transmembrane domain is 100% conserved among human, rat and mouse. Cys286 inside this region may be used for both plamitoylation regulating CD44 attachment to the cytoskeleton via ankyrin (Lokeshwar and Bourguignon, 1991) and disulfide bond formation between CD44 proteins, leading to dimerization (Liu and Sy, 1997). The 70 aa CD44 cytoplasmic domain shows more than 83% conservation of aa sequence among human, rat and mouse (Isacke, 1994; Sheikh et al., 1998). There are six serine residues in the cytoplasmic tail of human CD44, however, only Ser 323 and 325 are able to be phosphorylated *in vivo* (Peck and Isacke, 1996; Peck and Isacke, 1998). CD44 can associate with the actin cytoskeleton via interaction of cytoplasmic tail with ankyrin or the ERM family members (ezrin, radixin, and moesin). The ankyrin binding domain is mapped to a region between Asn304 and Leu318 (Lokeshwar et al., 1994). A region close to the cell membrane (Lys298-Lys300) comprises the ERM protein binding site (Legg and Isacke, 1998; Yonemura et al., 1998).

Insertion of variant exon sequences occurs downstream of aa residue 222. The CD44 variable region containing all variant exon sequences has four additional N-glycosylation sites and a number of O-glycosylation sites (Bennett et al., 1995). In addition, exon v3 has a consensus motif SGSG (Ser-Gly-Ser-Gly) for glycosaminoglycan modification (Jackson et al., 1995).

1.2 CD44 ligands

CD44 binds to several molecules, such as serglycin, addressin and osteopontin. Most importantly many components of the ECM, including collagen, fibronectin and proteoglycans have all been shown to be the CD44 ligands. One particularly interesting ligand of CD44 is HA.

1.2.1 HA

1.2.1.1 Introduction

HA is the largest ECM component with a molecular mass of up to several million daltons. Structurally it is a polymer of repeating disaccharide units with the structure (D-glucuronic acid [1-β-3]N-acetyl-O-glucosamine [1-β-4]. HA molecules of 10,000 repeats could extend to 10 μm length if stretched, however, in physiological solutions HA molecules are found in an expanded random coil structure in which extensive internal hydrogen bonds are formed. HA is synthesized at the inner face of the plasma membrane as a free linear polymer, in contrast to other glycosaminoglycans which are synthesized by resident Golgi enzymes and are covalently attached to protein cores. The HA synthases are integral plasma membrane glycosyltransferases that are proposed to coordinately polymerize and translocate HA out of the cell into the ECM (Itano et al., 1999). Mammalian cells are able to produce HA by using three HA synthases (Has). Has1 or 2 gives rise to high molecular weight HA whereas Has3 has a prepondence towards the biosynthesis of lower molecular weight HA. In mammals, there exist three groups of enzymes, namely hyaluronidase, β-D-glucuronidases and β-N-acetyl-D hexosaminidases, that are able to degrade HA.

1.2.1.2 HA functions

HA functions both as structual ECM organizer and extracellular signaling molecule. HA is able to bind salt and water, thereby expanding the extracellular space. HA participates in building up the three-dimensional structure of the ECM via associating with hyalectin, a class of proteoglycans. This HA-hyalectin interaction can further be stablized by small and globular proteins from the link protein superfamily. HA absence resulting from the gene disruption of Has2 in knock-out mice led to major reduction in cell free, matrix-defined spaces throughout day E9.5 p.c and embryonic lethality at E10.5 (Camenisch et al., 2000). In vitro, HA reduction due to blocking of Has2 expression also caused a decrease in the diameter of the cell-associated matrix (Nishida et al., 1999).

Like other ECM components, HA can act as a signaling molecule as well. This can be deduced from several experiments that induce HA synthesis upon transfection of either of the HA synthases. Has1 overexpression in a mouse mammary cancer cell line with low levels of HA synthesis increased its metastatic ability (Itano et al., 1999). Transfection of a human fibrosarcoma cells with Has2 enhanced both anchorage-independent growth and tumorigenecity (Kosaki et al., 1999). Has3, the most active isoform of the three enzymes, promoted the growth of prostate cancer cells upon overexpression (Liu et al., 2001). Furthermore, cardiac explants that were isolated from Has2 deficient E9.5 mouse embryos exhibited a profound defect in epithelial-mesenchymal transformation and cell migration on collagen gels (Camenisch et al., 2000). This defect was rescued by expression of wild-type Has2 or administering exogenous HA or expressing activated Ras, revealing a new pathway for cell migration and invasion that is HA-dependent and involves Ras activation.

1.2.1.3 Structural basis of CD44 binding to HA

HA belongs to the GAG family the members of which interact with their receptors via positively charged basic amino acids (Jackson et al., 1991). Such basic amino acids were also found to be important for HA binding of CD44. Mutations of three basic residues in the link module of CD44 reduced HA binding. While R29A and K38A mutations partially impaired binding of CD44-Immunoglobulin fusion proteins to immobilized HA, R41A (Arg was mutated to Ala) caused almost complete loss of binding (Peach et al., 1993). Based on the structure of the link domain of another link protein family member TSG-6, a molecular model of the CD44 link module was constructed, in which residues Arg41, Tyr42, Arg78 and Tyr79 form the center for HA binding which runs along a ridge on the protein surface (Bajorath et al., 1998). The putative surface of the HA binding center is broad and extensive, consistent with the minimal size of the HA ligand, a hexasaccharide (Underhill et al., 1983).

1.2.1.4 Regulation of CD44-mediated HA binding

The binding of HA to CD44 is highly regulated. Although all cells that express any form of CD44 on the cell surface bear the same conserved amino terminal sequence containing the HA recognization domain, many do not bind HA constitutively (Lesley and Hyman, 1998). Three activation states of CD44 have been identified in different cell lines. Cells expressing CD44 in an active state, can bind HA constitutively. Other cells do not bind HA constitutively, but can do so upon binding of certain CD44 specific mAbs or treating cells with stimuli such as phorbol ester and cytokines. In some cells CD44 can not bind HA at all even in the prescence of inducing mAb or cell stimuli. The inclusion of variant exons, different glycosylation patterns and clustering on cell surface can affect the HA binding capacity of CD44 molecules.

A. Binding of HA to CD44 variant isoforms

Different CD44 variant (CD44v) isoforms display different HA binding properties. CD44v7-10, v6-10. v3-10 and v3,8-10 failed to confer on Namalwa cells the ability to bind efficiently to HA in constrast to the dramatic enhanced HA binding of CD44s transfectants (Bartolazzi et al., 1995; Stamenkovic et al., 1991; van der Voort et al., 1995). A rat pancreatic adenocarcinoma cell line transfected with CD44v4-7 or CD44v6,7 demonstrated enhanced binding to both soluble and immobilized HA, whereas CD44s transfectants failed to bind (Sleeman et al., 1997; Sleeman et al., 1996). Even the HA binding of the same CD44v isoform has been reported to be variable. When mouse CD44v8-10 was introduced into a CD44 negative AKR1 cell line of mouse T-cell lymphoma, the transfectants exhibited significant binding to an HA-bearing cell line (He et al., 1992). However, a number of other reports pointed out that CD44v8-10 transfectants did not significantly bind to HA when it was transfected into a CD44 negative Burkitt´s lymphoma cell line Namalwa and a melanoma cell line (Bennett et al., 1995; van der Voort et al., 1995). Endothelial cells from different origins all contained CD44 v10 isoforms which were responsible for adhesion to HA (Lokeshwar et al., 1996). Conversely, overexpression of CD44 v10 nearly abolished binding of a human breast epithelial cell line which express endogenous CD44s (Iida and Bourguignon, 1997). These seemingly conflicting results can be reconciled by assuming that the specific cellular environment influences the ability of the CD44

variants to interact with HA. The prescence of variable exon products in CD44 provides additional glycosylation sites, therefore, it is likely that different cells synthesize CD44v molecules with distinct glycosylation patterns which may cause variable HA binding.

B. The effect of glycosylation on HA recognition

CD44 proteins undergo diverse glycosylation including N-glycosylation, O-glycosylation and GAG modification during the maturation process. CD44 from cells binding HA constitutively migrated more rapidly than CD44 from cells binding HA inducibly, which in turn migrated more rapidly than CD44 from cells unable to find HA, suggesting that glycosylation may negatively regulate HA binding property of CD44 (Lesley et al., 1995). Tunicamycin treatment induced the HA binding ability of cells, implicating N-glycans involved in the negative regulation of HA binding (Katoh et al., 1995; Lesley et al., 1995). The increase in HA binding by inhibition of N-glycosylation can also be achieved by treating cells with cytokines (Cichy and Pure, 2000; Hathcock et al., 1993). However, there are also contrary reports showing that the addition of N-glycans is indispensible to (Sleeman et al., 1996) or is not required for HA binding in different types of cells (Bartolazzi et al., 1996). Since deglycosylation of CD44 upon treatment with inhibitors of glycosylation affects not only the CD44 receptors but also other cell surface glycoproteins, a more direct approach, namely site-directed mutation of residues for N-glycosylation was used. Again, cell specific effects of glycosylation was observed. There are five N-glycan addition sites in the hematopoietic isoforms of CD44s. Mutation of any one of these sites abolished adhesion to immobilized HA in a human melanoma cell line (Bartolazzi et al., 1996). In contrast, the same mutations had no effect on constitutive HA binding in a HA-binding active lymphoma cell line (English et al., 1998), while inactivation of either the first or the fifth N-glycosylation site altered the inducible HA binding status of a lymphoma cell line to an active binding status. Removal of N-terminal sialic acid by treating cells with neuraminidase mostly enhanced HA binding ability (English et al., 1998; Katoh et al., 1995). This effect of sialic acid has been

confirmed also by other reports using different methods (Katoh et al., 1999; Skelton et al., 1998).

C. The effect of clustering on HA recognition

The first suggestion that cells might regulate HA binding of CD44 by clustering of CD44 on the cell surface was deduced from the following experiment: a CD44-specific mAb, IRAWB14, induced HA binding in cells otherwise did not bind HA (Lesley et al., 1993). HA binding was induced either upon treatment with the intact mAb or a multivalent Fab fragment, but not with a monovalent Fab fragment of the same mAb. In another experiment, a CD44s mutant, in which the transmembrane domain of CD44 was substituted by that of the CD3ζ chain, was expressed on the cell surface of a lymphoma cell line (Perschl et al., 1995). The CD3ζ domain enforced the formation of oligomers of the chimeric proteins. The cells expressing such chimeras bound HA much better than the cells expressing wild-type CD44s, suggesting that CD44 oligomers on the cell surface favor HA binding. Furthermore, the phorbol ester (PMA)-induced HA binding in a human Jurkat leukemia cell line was accompanied by PMA-induced clustering of CD44s (Liu and Sy, 1997). The clustering depended on a cysteine in the transmembrane domain (Cys286) of CD44s and a mutation of this Cys into Ala abolished HA binding. Interestingly, various isoforms of CD44 seem to differ regarding their clustering potential in the same cell line. Upon overexpression in a pancreatic carcinoma cell line, CD44v4-7 formed dimer and oligomer, whereas CD44s did not (Sleeman et al., 1996). The HA binding was correlated with HA binding because only the cells that formed oligomer could bind to HA.

1.2.2 Serglycin

Among GAGs, HA is the only free GAG, while other GAGs are covalently attached to protein cores to form proteoglycans (Ruoslahti, 1989). Besides binding to HA, CD44 can also bind to proteoglycans via association with their GAG side chains. One of such proteoglycans is serglycin. Serglycin is a secreted proteoglycan which is expressed in the yolk sac, in hematopoietic cells and in some tumor cells (Kolset and

Gallagher, 1990). Serglycin is encoded by a single gene and its peptide core is composed primarily of tandem serine-glycine repeats (Toyama-Sorimachi et al., 1995). The heterogeneity in the molecular weight of serglycerin (60-750 kD) is due to different use of residues for GAG addition, resulting in different GAG modification. CD44 interacts with serglycin through association with chondroitin 4-sulfate and/or chondroitin 6-sulfate side chains (Toyama-Sorimachi et al., 1997). The serglycin binding domain of CD44 is similar to or identical with the HA binding domain deduced from blocking of binding by the same antibody. Serglycin is produced and secreted by many cell types of hematopoietic origin, suggesting that interaction of serglycin with CD44 could be important in hematopoiesis. Indeed, binding of CD44 on a mouse cytotoxic T cell line (CTLL2) to serglycin augmented the CD3-dependent degranulation.

1.2.3 Aggrecan and versican

Two ECM proteoglycans, aggrecan and versican, were recently found to be able to bind to CD44. Aggrecan is a major component of the cartilage matrix and contains a core protein encoded by a single gene. The core protein has two amino terminal globular domains, G1 and G2, and a carboxyl terminal globular domain, G3 (Schwartz et al., 1999). Most of the keratan sulfate (KS) and chondroitin sulfate (CS) side chains are attached to a segment between G2 and G3. Soluble CD44-immunoglobulin G fusion proteins (CD44-IgG) and CD44-expressing cells bound to the aggrecan from rat chondrosarcoma and bovine cartilage in a manner dependent on CS side chains of aggrecan (Fujimoto et al., 2001). Structural analysis showed that the CS side chains of these rat and bovine aggrecans consist of mainly CSA and a mixture of CSA and C respectively. Versican is another large proteoglycan that exists in ECM. A versican derived from a renal adenocarcinoma cell line is modified with at least CSB and CSC (Kawashima et al., 2000). Similar to serglycin and aggrecan, the binding of CD44 to this versican was mediated by the interaction of the link module of CD44 with the CS chain of versican. Since soluble CD44 directly bound to immobilized HA, chondroitin, CSA, B, C, D, and E, it was predicted that other

proteoglycans besides serglycin, aggrecan and versican should be able to bind CD44 provided that they are sufficiently modified with the appropriate GAGs.

1.3 Tumorigenesis

1.3.1 Introduction

Cancer is one of the leading causes of human death in industrial countries. Tumorigenesis is a multistep process and the individual steps reflect genetic alterations in somatic cells that drive the progressive transformation of normal cells into highly malignant derivatives (Klein, 1998; Yokota, 2000). This concept is based on epidermiological studies of human cancers, cell culture studies and animal models. Epidemiological data demonstrate that the incidence for most types of human cancers increases exponentially with age, suggesting the necessary accumulation of mutations with age in tumor progression (Vogelstein and Kinzler, 1993). Pathological analysis of a number of samples from distinct, well-characterized morphological stages of cancer reveals a series of mutations that commonly arise in a well-defined order. For instance, during the development and metastasis of human colorectal cancer, sequential mutations including loss of active APC protein, activation of the K-ras protein, loss of expression of DCC, p53 proteins and other changes drive normal colon cells to form in turn polyps on the colon wall, then benign, later precancerous tumor, lesions that are eventually converted into metastatic adenocarcinoma (Vogelstein and Kinzler, 1993). Several alterations are required for the transformation of cells in tissue culture. For instance, the combination of the ectopic expression of the telomerase catalytic subunit (hTERT), the simian virus 40 large-T oncogene and H-ras oncogene is the minimal requirement for transforming human mammary epithelial cells (Elenbaas et al., 2001), embryonic kidney cells and foreskin fibroblasts (Hahn et al., 1999). In animal models, for example, overexpression of Myc in transgenic mice is insufficient to induce fast tumor formation, although overexpression of Myc protein is associated with many types of cancers (Sinn et al., 1987). Mammary tumors arise much more rapidly in double-transgenic mice that express both Myc and Ras than in mice expressing either Myc or Ras alone,

suggesting that these two somatic mutations must synergize to promote tumorigenesis.

A succession of genetic changes, each conferring one or another type of growth advantage, lead to the progressive conversion of normal human cells into cancer cells *in vivo*. These genetic changes are observed in six essential features that collectively dictate malignant growth: autonomy of growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, unlimited replicative potential, sustained angiogenesis, and programs for tissue invasion and metastasis (Hanahan and Weinberg, 2000).

1.3.2 Autonomy of growth signals

Normal cells proliferate upon contact with growth signals such as diffusible growth factors (GFs) or ECM components. Tumor cells generate their own growth signals, thereby reducing dependence on exogenous growth stimulation. For achieving this kind of autonomy, tumor cells adopt three common molecular strategies. The first involves alteration of extracellular growth signals. For instance, the v-sis oncogene of simian sarcoma virus (SSV) is a retroviral homolog of the PDGF B-chain gene. Infection of animals with SSV leads to induction of gliomas in which the PDGF receptors is activated in an autocrine fashion thereby resulting in unrestricted growth of the cells (Heldin, 1992). Secondly, cell surface receptors that transduce growth stimulatory signals into the cells themselves can be altered either in structure or in amount. For example, point mutation that changes a valine to glutamine in the transmembrane region of the Her2 receptor causes dimerization of two Her2 receptors in the absence of EGF ligand, making Her2 constitutively active (Yarden and Ullrich, 1988). The overexpression of the Her2 receptor as found in many human breast cancers results in stimulation of proliferation at very low concentrations of EGF or related hormones, concentrations that are too low for stimulation of proliferation of normal cells. The last and most frequent strategy that tumor cells use derives from alterations in components of the intracellular signaling pathway. Many proto-oncogenes encode proteins that act as intermediates in signal transduction cascades. Oncogenes can derive from proto-oncogenes by mutations. For instance, mutation

leading to the substitution of glycine at position 12 by another aa makes Ras protein oncogenic (Feig, 1993). The mutation reduces the protein's GTPase activity, thus rendering Ras constitutively active.

1.3.3 Insensitivity to antigrowth signals

Within a normal tissue, several soluble or immobilized growth inhibitors operate to maintain cellular quiescence and tissue homeostasis. Incipient cancer cells must evade these signals. One such signal is TGF_β that binds to its receptor and activates the synthesis of a protein (p15) which blocks the cyclin:CDK complexes that accounts for Rb phosphorylation (Hannon and Beach, 1994). Upon phosphorylation, Rb loses its ability to block proliferation by sequestering and inhibiting the function of E_2F transcription factors that control the expression of genes essential for progression through G1 phase (Weinberg, 1995). Some human tumor cells lose TGF_β responsiveness through downregulation of their TGF_β receptors, while others display mutant receptors that are dysfunctional (Fynan and Reiss, 1993; Markowitz et al., 1995).

1.3.4 Evading apoptosis

Tumor growth is determined not only by the rate of cell proliferation but also by the rate of cell death. Cancer cells can acquire resistance against apoptosis by overexpression of anti-apoptotic factors or loss of proapoptotic regulators. Members of the Bcl-2 protein family are the regulators of cell survival. One member of this family, Bcl-2, is an anti-apoptotic factor. Bcl-2 was originally found to be upregulated via chromosomal translocation in follicular lymphoma (Korsmeyer, 1992). The oncogenic potential of Bcl-2 was also observed with Bcl-2 transgenic mice (Adams and Cory, 1998). Bcl-2 synergizes with Myc in tumorigenesis, as documented for lymphoma and for breast cancer formation in double-transgenic mice. The most frequently occuring inactivation of a proapoptotic regulator is the loss of function of the p53 tumor suppressor gene. Inactive p53 is observed in more than 50% of human cancers (Harris, 1996). The p53 tumor suppressor protein can elicit apoptosis by

upregulating expression of the proapoptotic protein Bax which in turn stimulates mitochondria to release cytochrome C.

1.3.5 Unlimited potential for replication

Normal human cells have a limited capacity for doublings (60-70 rounds). The ends of their chromosomes (the telomeres), are shortened with each doubling (Counter et al., 1992) and this progressive shortening of telomeres finally results in the loss of protection of the ends of chromosomal DNA, end-to-end fusions of chromosomes and cell death. All types of malignant cells have the ability of telomere maintenance (shay and bacchetti,1997). 85%-90% of them succeed in doing so by upregulating expression of telomerase enzyme (Bodnar et al., 1998), which adds hexanucleotide repeats to the ends of telomeric DNA (Bryan and Cech, 1999). The importance of telomere maintenance for cancer also comes from analysis of mice lacking telomerase function. If the mice that are tumor prone are crossed with the telomerase knockout mice, upon carcinogen exposure, these double-knockout mice showed reduced tumor incidence (Greenberg et al., 1999).

1.3.6 Sustained angiogenesis

In order to progress to a larger size, tumors need nutrition support from blood. For that purpose they have to develop new blood vessels in the tumor tissue. The new blood vessel growth can be achieved either by upregulation of angiogenic factors such as VEGF (vascular endothelial GF) and FGF1/2 (fibroblast GF 1/2) or block of anti-angiogenic factors such as thrombospondin-1 (Bouck et al., 1996; Hanahan and Folkman, 1996). For example, tumor cells activate VEGF function either by activation of VEGF gene transcription or by enhancement of VEGF release. Upregulation of VEGF expression is induced by the VHL tumor suppressor gene in a number of tumor cell lines (Maxwell et al., 1999). VEGF release by MMP-9 is important for angiogenesis in a mouse model of pancreatic islet tumors (Bergers et al., 2000) and bFGF (basic FGF) stored in the ECM can be released by a variety of

proteases in tumor cells (Whitelock et al., 1996).

1.3.7 Invasion and metastasis

Metastasis is the cause of 90% human cancer deaths (Sporn, 1996). Genetic and biochemical determinants of metastasis remain incompletely understood. It is known that successful metastasis of cancer cells depend on all of the other above-mentioned five prerequisites. But additionally, metastasis utilizes strategies involving changes in the activation of extracellular proteases and in the physical coupling of cells to their microenvironment (Hanahan and Weinberg, 2000). The latter event involves changes of the function of cell adhesion molecules (CAMs). These CAMs include molecules mediating cell-cell adhesion such as E-cadherin and molecules mediating cell-ECM adhesion such as integrin and CD44. Proteases and CAMs are currently under extensive investigation and accummulating data strongly demonstrate that these molecules are closely involved in tumor cell proliferation, apoptosis, angiogenesis and metastasis. Here we focus on the role of these molecules in tumor metastasis.

1.3.7.1 Proteases:

Degradation of ECM by proteolysis can facilitate the metastasis of cancer cells (Werb, 1997). Most ECM degradation is accomplished by matrix metalloproteinases (MMPs) and serine proteinase, which are universally expressed during tumor progression and metastasis.

A. MMPs

The MMPs comprise a large family of over 20 proteins that can proteolyse all the known protein components of ECM (Nagase and Woessner, 1999). These proteinases demonstrate selectivity towards substates such that an individual MMP has the unique ability to degrade a particular subset of matrix proteins, corresponding to the complex functions in which each MMP is involved.

The expression, secretion and activity of MMPs are strictly regulated (McCawley and Matrisian, 2000). For instance, MMPs are secreted in an inactive latent form that

requires cleavage of peptide bond to attain the catalytically active state. This activation of MMPs is regulated by proteinases. MMP activity is further modulated by their natural inhibitors, the tissue specific inhibitors of matrix-metalloproteinases (TIMPs). Deregulation of MMPs has been widely linked to tumor invasion and metastasis. For example, the upregulation of MMP-2 and MMP-3 is related to lymph node metastasis and vascular invasion in squamous cell carcinoma of the oesophagus (Shima et al., 1992). Increased MMP-11 expression is associated with increased invasiveness in head and neck squamous cell carcinomas (Muller et al., 1993). Indeed, MMP levels serve as a prognostic factor in multiple tumor types (Stetler-Stevenson et al., 1996). An important role of MMPs in tumor invasion and metastasis has also been confirmed by experimental models. For example, overexpression of MMP-3 in mouse mammary tumor cells accompanies acquisition of MMP-3-dependent invasive properties (Lochter et al., 1997). In an *in vivo* study of mammary carcinogenesis, MMP-3 expression not only promotes the tumor formation at the onset stage, but also confers the invasive phenotype to mammary epithelial cells (Sternlicht et al., 1999). Overexpression of TIMP-1 or TIMP-2, which downregulate the activity of MMPs by directly binding the catalytic site of MMPs, inhibits invasion, metastasis and tumorigenecity of malignant cells (DeClerck et al., 1992; Khokha, 1994; Khokha et al., 1992).

B. Serine proteinases

Serine proteinase is another group of extracellular protelytic enzymes that include thrombin, tissue plasminogen activator (tPA), urokinase (uPA) and plasmin (Werb, 1997). tPA, uPA and plasmin belong to the urokinase-type plasminogen activator system (Andreasen et al., 1997). As a downstream effector of this system, plasmin is converted from the inactivate zymogen plasminogen. Such conversion is regulated by two types of plasminogen activators (uPA and tPA), two main inhibitors of plasminogen activators (PAI-1 and PAI-2) and uPA receptor (uPAR).

uPA plasminogen activator system is strongly involved in cancer metastasis, based on the results from many animal model systems and human cancers (Andreasen et al., 1997). uPA-catalyzed plasminogen activation is rate-limiting for local tumor invasion

and formation of distant metastasis. A correlation between the uPA expression and lung metastasis of a series of human melanoma cell lines was found in nude mice model (Quax et al., 1991). Antibodies against human uPA prevented local invasion of cancer cells (Ossowski et al., 1991). Transfection of a rat prostate carcinoma cells with uPA enhanced their metastasis potential *in vivo* (Achbarou et al., 1994). uPAR binding of uPA seems to be necessary for metastasis, because reduction of uPAR expression in implanted human squamous cancer cells by anti-sense approach reduced both invasion and metastasis in nude mice (Kook et al., 1994). Saturation of uPAR with an enzymatically inactive but uPAR-binding uPA variant also inhibited metastasis of primary tumors produced by inoculating a prostate carcinoma cell line (Crowley et al., 1993). In uPA activator system PAI-2 was reported to suppress lung metastasis spontaneously developed in mice injected with PAI-2 overexpressing human melonoma cells (Mueller et al., 1995). Clinically, uPA is the strong prognostic factor of certain cancers such as cancers of human breast, lung, bladder, stomach, and colorectum (Andreasen et al., 1997). The molecular mechanism of uPA system-involved tumor invasion and metastasis can not just be ascribed to the proteolytic activity of uPA, other additional mechanisms also exist (Murphy and Gavrilovic, 1999). Except binding to uPA, uPAR also functions as an adhesion receptor for vitronectin and interacts laterally with integrin β chains on plasma membrane. PAI-1 blocks the binding between vitronectin and uPAR-uPA or between vitronectin and integrins. Therefore, the functional analysis of the interaction of uPA with integrins, which are also implicated in metastasis formation, may provide better understanding of the action of uPA in tumor progression.

1.3.7.2 Integrins

Integrins comprise a large family of cell surface receptors that link cells to their microenvironment. They are composed of two subunits, α and β, and each αβ combination has its own ECM protein ligands and its own signaling properties (Giancotti and Ruoslahti, 1999). Cancer cells facilitate invasion by shifting their expression of integrins from those that favor the ECM present in normal epithelium to

other integrins that preferentially bind other set of ECM ligands (Lukashev and Werb, 1998; Varner and Cheresh, 1996). For example, overexpression of the $\alpha_2\beta_1$ integrin in a rhabdomyosarcoma cell line increased its metastatic potential (Chan et al., 1991), and $\alpha_v\beta_3$ integrin promoted the pulmonary metastasis of melanoma cells (Filardo et al., 1995). Conversely, forced expression of integrins $\alpha_5\beta_1$ and $\alpha_3\beta_1$ inhibited the metastatic capacity of several tumor cell lines (Giancotti and Ruoslahti, 1990; Weitzman et al., 1996). Recently, several mechanisms by which integrins regulate invasion and metastasis have been reported. Integrin $\alpha_v\beta_3$ can localize a proteolytically active form of MMP-2 on the surface of invasive tumor cells or endothelial cells (Varner and Cheresh, 1996). This localization appears to provide migratory cells with coordinated matrix degradation and cellular motility, thus facilitating cellular invasion. Furthermore, the association with MMP has also been described for $\alpha_2\beta_3$, $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins, suggesting the importance of this mechanism in tumor invasion and metastasis (Varner and Cheresh, 1996). Exploration of integrin signaling provides evidence that integrins regulate cell spreading and migration which are prerequisites for tumor invasion and metastasis (Giancotti and Ruoslahti, 1999). Integrins can activate the Rho-family of small guanine nucleotide-binding proteins including Rho, Cdc42 and Rac, each of which controls the actin cytoskeleton and is tightly involved in cell migration process by interacting with multiple downstream effectors (Hall, 1998). Two major components of integrin signaling pathway, FAK (focal adhesion kinase) and Src, also play a role in cell migration, perhaps by promoting the disassembly of focal adhesions at the trailing edge of the cell (Cary et al., 1998).

1.3.7.3 E-cadherin

E-cadherin is ubiquitously expressed in epithelial cells and mediates Ca^{2+} dependent homophilic interactions among cells. Clinical and experimental studies have revealed that E-cadherin function is lost during the development of most, if not all, human epithelial cancers (Christofori and Semb, 1999). Using tumor cell lines in culture, several groups found that re-establishing the functional cadherin complex, for

example by forced expression of E-cadherin, resulted in a reversion from an invasive to a benign tumor cell phenotype (Christofori and Semb, 1999). In an *in vivo* tumor model in which mice developed pancreatic carcinoma upon expression of the SV40 large T antigen in pancreatic β cells, overexpression of E-cadherin during β-cell tumorigenesis led to arrest of tumor development at the benign adenoma stage. In contrast, inhibition of E-cadherin function induced tumor invasion and metastasis (Perl et al., 1998).

Two consequences result from the loss of E-cadherin function in cancers. One is the loss of cell-cell adhesion, which allows cancer cells to disseminate and invade surrounding tissue. The second consequence is the disruption of E-cadherin-mediated signaling processes that regulate gene expression and cell fate. β-catenin is part of the E-cadherin adherens junction complex. Loss of E-cadherin expression leads to the release of β-catenin which then translocates to the nucleus, binds to a member of the LEF-1 (leukocyte enhancer factor-1) family of transcription factors and activates the expression of target genes (Christofori and Semb, 1999). Among the target genes of LEF-1/β-catenin are MYC (He et al., 1998) and MMP-7 (Crawford et al., 1999) which themself seem to be involved in tumorigenesis.

1.4 CD44 and tumor growth and metastasis

1.4.1 CD44 function in tumorigenesis

In human cancers, CD44 has been demonstrated to be a diagnostic and prognostic factor (Naor et al., 1997). For example, expression of CD44 v3 or v6 are strong independent prognosticators in patients with colorectal cancer (Wielenga et al., 2000). Expression of CD44s or CD44 v6 is associated with poor prognosis in human non-Hodgkins's lymphoma (Pals et al., 1997) and increased CD44 v7/v8 expression is highly correlated with the progression of human cervical carcinoma (Dall et al., 1996).

Evidence from several experimental systems undoubtly implicates that CD44 molecules are decisive for tumor development. CD44 seems to be involved in tumor

growth and metastasis processes as demonstrated for pancreatic carcinoma, melanoma, mammary carcinoma and lymphoma.

CD44 v6-containing isoforms were the first CD44 variant isoforms that have been shown to be involved in tumor metastasis formation. The two CD44 variants CD44v4-7 and CD44v6,7 were identified on a rat pancreatic carcinoma cell line with metastatic capacity but were absent on its non-metastatic counterpart (Günthert et al., 1991). The expression of each of these isoforms in the non-metastatic pancreatic carcinoma cells sufficed to establish metastases in the lung and lymph node upon subcutaneous injection in syngeneic animals (Rudy et al., 1993). Administration of monoclonal Abs directed against a CD44 v6 epitope prior and together with injection of metastatic pancreatic tumor cells inhibited outgrowth of lung and lymph node metastase, clearly demonstrating that CD44 v6-containing variant isoforms play an important role in the metastatic process (Seiter et al., 1993).

In melanoma cell lines the relevance of CD44 in tumorigenesis was deduced by comparing the metastatic features of two human melanoma cell lines with high or low levels of CD44 expression obtained from one parental cell line. The intravenous injection of these two cell lines each into nu/nu BALB/c mice revealed that the CD44 high expressors developed significantly more lung nodules than the low expressors (Birch et al., 1991). In another study, the human melanoma cell line SMMU-2 with high expression levels for CD44 was established from lymph node metastases (Guo et al., 1994), while SMMU-1 with low levels of CD44 expression was obtained from a primary tumor. Upon subcutaneous injection into nu/nu mice, only SMMU-2 developed metastases, again suggesting a decisive role for CD44 in metastatic spreading. Furthermore, a monoclonal antibody directed against human CD44 inhibited local tumor growth and metastatic spreading of SMMU-2 tumor cells. In a third melanoma system, CD44 was transfected into CD44-negative melanoma cell lines (Bartolazzi et al., 1994). Overexpression of CD44s, but not of the CD44v8-10 isoform in transfected cells, resulted in tumor growth upon subcutaneous injection into nu/nu mice. Interestingly, this tumor growth could be inhibited by local injection of a soluble CD44-Ig fusion protein which may compete with cell-surface localized CD44 for ligand binding.

For mammary carcinoma the relevance of CD44 for metastasis formation was proven by expression of soluble CD44 in a metastatic murine mammary carcinoma cell line which endogenously expressed several CD44 isoforms (Yu and Stamenkovic, 1999; Yu et al., 1997). In experimental metastasis assays, the formation of pulmonary metastases were completely inhibited when cells expressing soluble CD44 were used, no matter whether CD44s, CD44v6-10 or CD44v8-10 was used as soluble forms.

Several investigations of various functional aspects of CD44 isoforms were performed with a human Burkitt's lymphoma cell line (Namalwa) which does not express any CD44 isoform. Transfection of CD44s into these cells enhanced local tumor growth, which was inhibited by a soluble human CD44s-Ig fusion protein (Bartolazzi et al., 1995; Sy et al., 1991; Sy et al., 1992). Among the CD44 variant isoforms, CD44v8-10 was able to suppress subcutaneous Namalwa growth when expressed in Namalwa cells, while CD44v7-10, v6-10, v3-10 and CD44v3,8-10 either had inhibitory or no effect on tumor growth (Bartolazzi et al., 1995; Sy et al., 1991; Yakushijin et al., 1998). Regarding the effect of CD44 on metastasis, CD44s expression supported metastatic spreading while CD44v6-10, v7-10, v8-10 retarded metastasis formation when expressed on Namalwa cells (Bartolazzi et al., 1995; Yakushijin et al., 1998). CD44v3-10 and CD44v3,v8-10 were reported to increase bone marrow metastasis (Bartolazzi et al., 1995), in constrast to this, CD44v3-10 was found to inhibit metastasis in another report (Yakushijin et al., 1998). Interestingly, CD44v6 isoform, the epitope of which is also expressed by normal activated human lymphocytes (Arch et al., 1992), was shown to be able to enhance both the local tumor growth and experimental metastasis of Namalwa cells (Yakushijin et al., 1998). CD44 seemed to be also involved in tumorigenesis of another lymphoma cell line. The LB cell line is a murine T cell lymphoma cell line that was isolated from a lymphoma spontaneously developed in a BALB/c mouse strain (Zahalka et al., 1993). When injected subcutaneously into syngeneic mice, LB lymphoma cells invaded into spleen and peripheral lymph nodes. The invasion was dependent on two types of cell-surface molecules, namely LFA-1 (integrin $\alpha_L\beta_2$) and CD44s. Spleen invasion of LB cells was integrin-dependent, whereas invasion into lymph nodes could be blocked by anti-pan CD44 Ab (Zahalka et al., 1995). Furthermore, LB cells transfected with

CD44v4-10 grew faster and infiltrated more aggressively into lymph node than CD44s transfectants and parental cells (Naor D., personal communication).

1.4.2 Mechanism of CD44 function in tumor growth and metastasis

Data from the analysis of human tumor and experimental models demonstrate that CD44 plays a role in tumorigenesis, however, the molecular mechanism of the CD44 function remains rather obscure. Even for one of the main functions of CD44 isoforms, namely their ability to bind to HA, contrary results were obtained in different systems regarding its relevance to tumor formation. For example, in animal models, the CD44-HA interaction is a decisive factor in melanoma tumor development (Bartolazzi et al., 1994; Birch et al., 1991; Zhang et al., 1995). Expression of a wild-type CD44s in a melanoma cell line enforced HA binding and tumor formation in vivo, whereas expression of a CD44s mutant, which was unable to mediate cell attachment to HA, failed to do so. Furthermore, local administration of a CD44-Ig fusion protein defective in HA binding had no effect on subcutaneous melanoma growth in mice, while wild-tpye CD44-Ig blocked tumor development. These were examples for the importance of HA binding in tumorigenesis, but in a rat pancreatic carcinoma the CD44-HA interaction appeared irrelevant for tumor formation. Transfection of CD44 v6,7 into non-metastatic cells conferred metastatic spreading upon injection of cells into syngeneic animals. HA binding of these cells was prevented by simultaneous expression of human hyaluronidase on the surface. The hyaluronidase degraded effectively HA, but its expression did neither interfere with tumor growth nor metastasis formation (Sleeman et al., 1996).

CD44 can influence tumorigenesis by induction of cell migration and/or proliferation upon binding to HA. Migration appears to be controlled via activation of Rho GTPase upon HA binding. The Rho GTPase belongs to the Ras superfamily of small guanosine triphosphatases (GTPases) and is the key regulatory molecule that links surface receptors to the actin cytoskeleton (Hall, 1998). Typically, the activation of three GTPases RhoA, Rac1 and cdc42 leads to formation of stress fiber, focal adhesion plaques, lamellipodia, filopodia, microspikes and membrane ruffling in fibroblasts. In a mouse mammary epithelial cell line, HA binding to CD44 led to Rac1

activation and the formation of lamellipodial protrusions. These protusions were not observed when dominant-negative Rac was expressed or when the cells were pretreated with anti-CD44 antibodies (Oliferenko et al., 2000). A similar contribution of CD44-HA interaction to Rac activation was observed in a murine metastatic breast tumor cell line. Tiam1 (T lymphoma invasion and metastasis 1) (Habets et al., 1994), one of the Rac-specific guanine nucleotide exchange factors (GEFs), associated with CD44v3,8-10 as a complex *in vivo* (Bourguignon et al., 2000). HA binding to CD44v3,8-10 activated Rac1 signaling via Tiam1 and enhanced cytoskeleton-mediated cell migration.

In a human ovarian tumor cell line another GEF, Vav2, was also shown to associate with CD44v3 both *in vivo* and *in vitro* (Bourguignon et al., 2001). The binding of HA to CD44v3 activated Vav2-mediated Rac1 signaling leading to ovarian tumor cell migration. In this system the HA-CD44 interaction seems to contribute to cell migration also through a second mechanism. CD44s and the Src kinase established a complex in the same cell line (Bourguignon et al., 2001). Upon HA binding to CD44s, Src catalyzed tyrosine phosphorylation of the cytoskeletal protein cortactin which then crosslinked filamentous actin and enhanced tumor cell migration.

HA-CD44 interaction can generate growth signals in tumor cells. For example, CD44 could crosstalk with the growth factor receptor p185HER2 (Bourguignon et al., 1997; Bourguignon et al., 2001). HA binding to human ovarian tumor cells promoted recruitment of both p185HER2 and the adaptor protein Grb2 to CD44v3, leading to Ras activation and ovarian tumor cell growth. A dominative-negative Grb2 effectively inhibited HA/CD44v3-induced Rac1 and Ras signaling as well as tumor cell growth.

CD44 also promotes cell proliferation through binding and presenting growth factors (GFs) to their cognate receptors. Binding and presenting GFs by CD44 sometimes requires heparan sulfate (HS) modification of CD44 molecules. HS-modified CD44 isoforms overexpressed in COS cell transfectants were found to be able to bind basic fibroblast growth factor (b-FGF) and heparin-binding epidermal growth factor (HB-EGF) in an *in vitro* assay (Bennett et al., 1995). In the Namalwa lymphoma cell line, CD44 isoforms containing exon v3 were heparan sulfated and able to bind hepatocyte growth factor/ scatter factor (HGF/SF) in HS-dependent

manner (van der Voort et al., 1999). Binding of HGF by CD44 promoted HGF receptor (c-Met) activation and phosphorylation of ERK-1 and ERK–2. CD44v3,8-10 was also modified by HS and bound vascular endothelial growth factor (VEGF) in a murine breast tumor cell line (Bourguignon et al., 1998). In a transgenic mouse system downregulation of CD44 expression by antisense CD44 cDNA led to reduced keratinocyte proliferation in response to b-FGF and HB-EGF, indicating that CD44 might bind and present these GFs *in vivo* (Kaya et al., 1997). A CD44 variant was expressed in apical ectodermal ridge (AER) and was required for limb outgrowth (Sherman et al., 1998). HS was essential for CD44-mediated growth promotion in this system because only HS-modified CD44v3-10 expressed on Namalwa cells bound and presented both FGF-4 and FGF-8 and stimulated mesenchymal cells *in vitro*.

CD44 can regulate cell growth also via heparan-sulfate-independent mechanism. By using rat pancreatic carcinoma cell system, CD44 isoforms that were not modified with HS bound and presented HGF to c-Met, leading to ERK-1 and ERK–2 phosphorylation (Rousseau et al., unpublished results). Furthermore, heparinase treatment did abolish the HS modification but failed to compromise the growth factor presentation function of CD44 in a human colon carcinoma cell line. CD44 was required to organize a ternary complex containing c-Met, HGF/SF and CD44. By a different mechanism CD44 seems to contribute to transforming growth factor β (TGF-β) receptor activation. CD44 was shown to localize matrix metalloprotease 9 (MMP-9) on cell surface in a mammary tumor cell line (Yu and Stamenkovic, 1999). CD44-localized MMP-9 on cell surface proteolytically activated TGF-β, subseqently leading to TGF-β-mediated angiogenesis and tumor growth (Yu and Stamenkovic, 2000).

1.5 The aim of my project

The aim of my project was to study the role of CD44 variant isoforms in lymphoma development *in vivo* and the correspondent mechanisms by using the Tet-off gene expression system to conditionally express CD44 in LB cell line. To reach this goal, I intended to establish transfectants of the LB lymphoma cell line in which the expression of four CD44 isoforms, namely CD44v3-10, v4-10, v8-10 and CD44s

were controled by the Tet-off promoter. With this system, I wanted firstly characterize the binding ability of CD44 isoforms to hyaluronate and other glycosaminoglycans. Finally, I wanted to investigate CD44 function in lymphoma development by injection of LB transfectants into syngeneic and immunocompromised mice.

PART TWO

MATERIALS AND METHODS

2.1 Materials

All general chemicals were supplied from Merck (Darmstadt), Carl Roth GmbH & Co (Karlsruhe) and Sigma Chemie GmbH (Deisenhofen) and with highest purity grade. Dimerizer AP20187 was kindly provided by ARIAD Pharmaceuticals, INC (Cambridge, Massachusetts). HA (from rooster comb), chondroitin sulfate A, B, C, heparin, heparan sulfate, keratan sulfate (SIGMA) were dissolved in PBS (w/o Ca, Mg) and incubated at 95°C for 10 min. FITC-HA (labelled according to (de Belder and Wik, 1975)) was kindly provided by Dr. Moll.

2.1.1 Primers

All the primers were synthesized by MWG Biotech GmbH and were of HPSF purity grade.

Primers for constructing mCD44v3-10-D10-3:

For(Balv3): 5′-CCTTGGCCACCAGTACGGAGT-3′

Rev(Bclstandard): 5′-CCGTTGATCACCTTTTTCT-3′

Primers for constructing mCD44v8-10-D10-3:

v8-10For(BalExn8):

5′-CACCTTGGCCACCAATACAGACTCCAGTCATA-3′

Rev(Bclstandard):

5′-CCGTTGATCACCTTTTTCT-3′

Primers for constructing mCD44v8-10-D-10-3 (C286A):

C286A-5′:

5′-GCTCTGATTCTTGCCGTCGCAATCGCGGTCAATAGTAGG-3′

C286A-3′:

5′-CCTACTATTGACCGCGATTGCGACGGCAAGAATCAGAGC-3′

Primers for constructing mCD44v8-10-(3)FKBP-D10-3:

EcoRI-mCD44(FKBP): 5′-GGGGAATTCGACCCTTTTCCAGAG-3′

XbalI-mCD44(FKBP): 5′-GGGGTCTAGACACCCCAATCTTCATGTCCA-3′

Primers for sequencing CD44:

P1: 5′-TAGGCGTGTACGGTGGGAG-3′

P2: 5′-CTGAGCAAGGGTTTTGAAACA-3′

P3: 5′-CAGAAGGCTACATTTTGCACA-3′

P4: 5′-GGTTTCAGAACGGATGGCAG-3′

P5: 5′-TCCAACAACTTCTATTCTGCCA-3′

P6: GAGACCTCAGATTCCAGAATG-3

2.1.2 Plasmids

pcDNA3.1+ (Invitrogen); pC4Fv1E and pC4MFv2E (kindly provided by ARIAD Pharmaceuticals, INC); pEGFP-N1 (CLONTECH); mCD44s-pcDNA3.1 and mCD44v4-10-pcDNA3.1 were kindly provided by Dr. Hofmann; pUHD10-3, pUHC13-3 and pBabepuro were kindly conferred by Dr. Englert and pUHD15-1neo by Dr. Weih. pUHD10-3, pUHC13-3 and pUHD15-1neo were originally described by Gossen (Gossen and Bujard, 1992).

2.1.3 Constructs

2.1.3.1 Name and description

Name	Description
mCD44s-D10-3	mCD44s under the control of Tet-responsive element and minimal CMV promoter
mCD44v4-10-D10-3	mCD44v4-10 under the control of Tet-responsive element and minimal CMV promoter
mCD44v8-10-D10-3	mCD44v8-10 under the control of Tet-responsive element and minimal CMV promoter
mCD44v3-10-D10-3	mCD44v3-10 under the control of Tet-responsive element and minimal CMV promoter
mCD44v8-10-D10-3 (C286A)	mCD44v8-10 with C286A mutation under the control of

	Tet-responsive element and minimal CMV promoter
mCD44v8-10-(3)FKBP-D10-3	mCD44v8-10-(3)FKBP fusion protein under the control of Tet-responsive element and minimal CMV promoter

2.1.3.2 Construction of mCD44-D10-3

For the construction of mCD44s-D10-3 and mCD44v4-10-D10-3 plasmids, CD44s- and CD44v4-10-containing fragments were digested out of mCD44s-pCDNA3.1 and mCD44v4-10-pCDNA3.1 vectors respectively with *EcoR1* and subcloned into pUHD10-3 vector. mCD44v3-10-D10-3 was constructed firstly by PCR amplification of CD44v3-10 fragment flanked by *Bcl1* and *Bal1* sites from the cDNA which was reversely transcribed from mRNA of a murine mammary epithelial cell line (HC11). Then the fragment was ligated to the vector (mCD44v4-10-D10-3) digested with the same pair of restriction enzymes (Fig. 2A). mCD44v8-10-D10-3 was made in similar way except that the PCR fragment was amplified from mCD44v4-10-D10-3 (Fig. 2B).

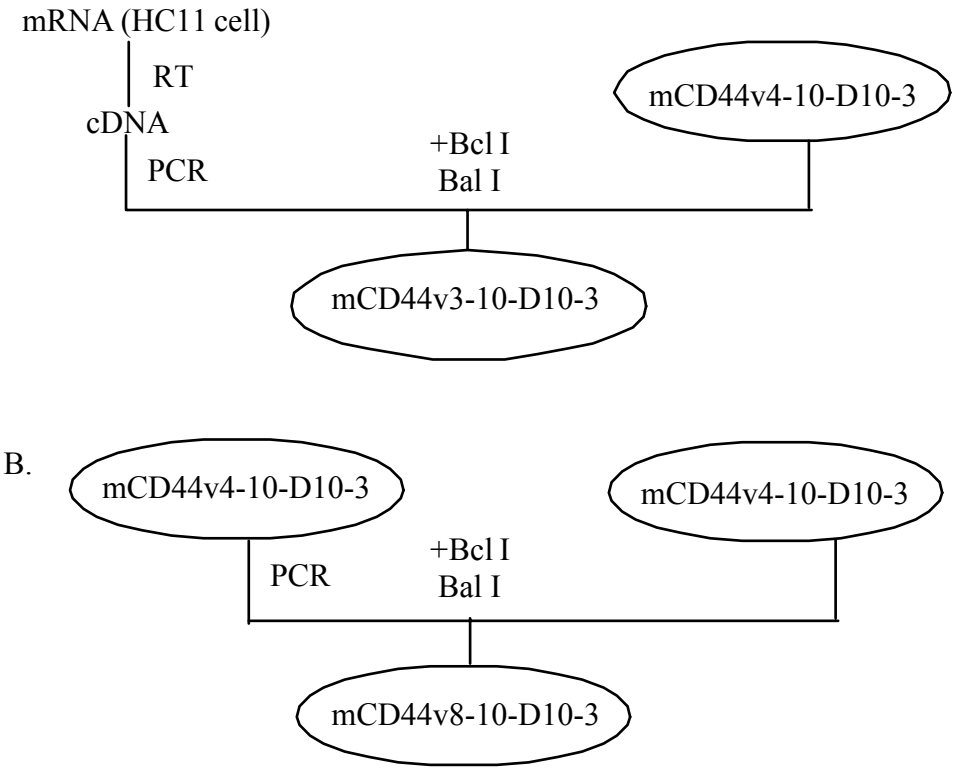


Figure 2. Schematic representation of contructing mCD44v3-10-D10-3 and mCD44v8-10-D10-3.

2.1.3.3 Construction of mCD44v8-10-FKBP and –3FKBP

One or three dimerization domains of a mutated FKBP sequence (F36v in which aa 36 has been mutated from phenylalanine to valine to avoid the reaction of FKBP to any endogenous ligand) and a hemagglutinin tag were fused to the cytoplasmic tail of CD44v8-10 to result in the CD44 fusion proteins: mCD44v8-10-FKBP and –3FKBP respectively (Fig. 3).

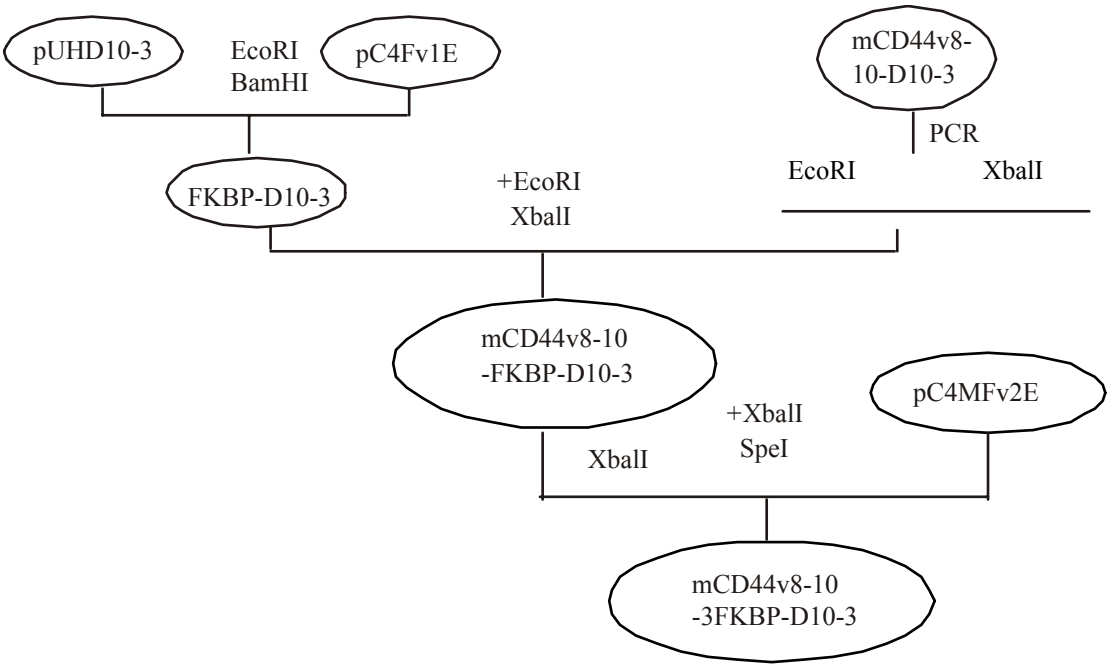


Figure 3. Schematic diagram of CD44v8-10-(3)FKBP-D10-3 construction.

All the constructs were sequenced before application.

2.1.4 Radiochemicals

[^{35}S] labelling mix (Amersham Life Science); [^{33}P] ddNTPs (Amersham Life Science).

2.1.5 Antibodies

Primary antibodies:

Name	Description	Supplier
IM7	rat IgG2b anti-mouse/human pan CD44 epitope	M. Hegen
KM81	rat anti-mouse CD44 pan CD44 epitope, blocking HA binding mediated by CD44	M. Hegen
10DI	rat anti-mouse CD44 v4	M. Hegen

LN10.1	rat anti-mouse CD44 v10	U. Günthert
FKBP	Mouse anti-human FKBP	PharMingen
12CA5	mouse anti-influenza hemagglutinin tag	ROCHE

Secondary antibodies:

Name	Description	Supplier
Rat HRP	rabbit IgG	Dako
Mouse HRP	rat IgG	Dako
Rat RPE	goat Ig(H+L)	PharMingen

2.1.6 Cell lines and media

HC11 cell line is a mouse mammary epithelial cell line (Ball et al., 1988) and was cultured in RPMI medium with 10% FCS and 5 μg/ml bovine insulin (SIGMA).

LB cell line is a mouse T cell lymphoma cell line (Zahalka et al., 1993) and was cultured in RPMI medium with L-glutamine, containing: 10% FCS, 1× non-essential amino acids, 10 mM Hepes pH 7.4, 1 mM sodium pyruvate, 1× streptomycin/penicillin, 50 μM β-ME (all from GIBCO BRL) and 0.5 mg/ml bovine insulin (SIGMA).

2.1.7 Mice

BALB/c mice and MF1 nude mice were kept under standard pathogen-free conditions.

2.2 Methods

2.2.1 Plasmid minipreparation

Individual colonies were picked from a LB agar plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% Agar and corresponding antibiotic) and inoculated into 3 ml of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing antibiotic. After overnight incubation at 37°C with shaking speed at 220 rpm, 1.5 ml of bacteria were pelleted by centrifugation at 13,000 g for 10 sec. The pellet was resuspended in 50 μl of solution I (50 mM glucose, 25 mM Tris·Cl, pH 8.0, 10 mM EDTA, pH 8.0), mixed

completely, and incubated on ice for 5 min. 100 µl of solution II (200 mM NaOH, 1 % SDS, made freshly) was then added and mixed gently by inverting the eppendorf tube for 6 times. After 5 min incubation, 75 µl solution III (3M sodium acetate, pH 4.8) was added, mixed gently and incubated on ice for 5 min. The protein-DNA complex was cleared off by centrifugation at 13,000 g for 3 min, and the supernatant was transferred to another eppendorf tube, mixed well with 2 volume of 100% ethanol, and incubated at room temperature for 5 min. The plasmid was finally recovered by centrifugation at 13,000 g for 1 min, washed with 70% ethanol once, and dissolved in 20 µl of TE (10 mM Tris·Cl, pH 8.0, 1 mM EDTA pH 8.0) containing RNase A (20 µg/ml).

2.2.2 Plasmid maxipreparation

According to the protocol from QIAGEN, 0.4 ml of LB medium containing bacteria (left from minipreparation) were inoculated into 400 ml of selective LB medium, and shaked vigorously for 14 hr. The bacteria were harvested by centrifugation at 6000 g for 15 min at 4°C. The pellet was resuspended in 10 ml of Buffer P1, and 10 ml of Buffer P2 was added, mixed gently but thoroughly. The mixture was incubated at room temperature for 5 min. 10 ml of chilled Buffer P3 was added, mixed and incubated on ice for 15 min. After centrifugation at 20,000 g for 30 min at 4°C, supernatant was removed and re-centrifuged for 15 min. The supernatant was applied to a pre-equilibrated QIAGEN-tip 500 and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2× 30 ml Buffer QC and DNA was eluted with 15 ml Buffer QF. DNA was precipitated by adding 10.5 ml room-temperature isopropanol to the eluted DNA fraction and centrifuging at 15,000 g for 30 min at 4°C. DNA pellet was washed with 5 ml of room-temperature 70% ethanol twice and centrifuged at 15,000 g for 10 min. After air-dried, the pellet was dissolved in 300 µl sterilized H_2O. DNA concentration was determined by UV spectrophotometry and calculated according to the following formula: 1 OD_{260}=50 µg/ml of double-strand DNA.

Composition of buffers:

Buffer	Composition
Buffer P1 (Resuspension Buffer)	50 mM Tris·Cl, pH 8.0;

	10 mM EDTA; 100 µg/ml RNase A
Buffer P2 (Lysis Buffer)	200 mM NaOH; 1% SDS (W/V)
Buffer P3 (Neutralization Buffer)	3.0 M potassium acetate, pH 5.5
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v) 0.15% Triton® X-100 (v/v)
Buffer QC (Wash Buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)
Buffer QF (Elution Buffer)	125 mM NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol (v/v)

2.2.3 Preparation of competent bacteria

One colony was inoculated into 3 ml LB medium without selection pressure and incubated overnight with shaking (220 rpm). 1 ml of this medium was diluted to 100 ml LB medium and incubated with shaking until OD_{550}=0.3-0.5. Bacteria were left on ice for 10 min after transferred to two 50 ml tubes and precipitated by centrifugation at 4000 rpm for 10 min at 4°C. The pellet in each tube was washed with 25 ml of chilled sterile $MgCl_2$ (100 mM) and recovered by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was resuspended in 5 ml chilled sterile $CaCl_2$ (100 mM), incubated on ice for at least 30 min and precipitated by centrifugation at 4000 rpm for 5 min at 4°C. Finally, all the bacteria were resuspended in 6 ml chilled $CaCl_2$/glycerol (4.8 ml 100 mM $CaCl_2$ plus 1.2 ml 86% glycerol), 400 µl of which were aliquoted to one eppendorf tube, snap-frozen in liquid nitrogen and stored at -80°C.

2.2.4 Polymerase Chain Reaction (PCR)

All PCR were performed in a total volume of 50 µl, in the presence of 10 ng template, 200 µM dNTPs, 0.2 µM of each primer, 1× reaction buffer, 10% DMSO and 1 µl pfu Turbo (STRATAGENE). The reactions were carried out in PCR machines (Perkin Elmer 9600/2400), using specific cycling parameters depending on the application.

2.2.5 RT-PCR (for constructing mCD44v3-10-D10-3)

2.2.5.1 Isolation of total RNA from cells

10^6 cells were pelleted by centrifugation and lysed in 1ml TRIzol Reagent (Gibco BRL) by repetitive pipetting. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per ml of TRIzol Reagent was added. The sample tubes were capped securely and shaked vigorously by hand for 15 sec. After 3 min incubation at room temperature, the samples were centrifuged at 12,000 g for 15 min at 4°C. Following centrifugation, the aqueous phase was transferred to a fresh tube, and the RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol per ml TRIzol Reagent used for the initial homogenization. Samples were kept at room temperature for 10 min and centrifuged at 12,000 g for 10 min at 4°C. 1 ml of 75% ethanol was added to the RNA pellet and the pellet was mixed by votexing and precipitated by centrifugation at 7,500 g for 5 min at 4°C. The RNA pellet was briefly dried at room temperature for 5 min and dissolved in 100 µl RNase free H_2O. 4 µl of the RNA was diluted to 400 µl and the concentration was measured.

2.2.5.2 Isolation of poly $(A)^+$ mRNA from total RNA

Total RNA no more than 250 µg was pipeted into an RNase-free 1.5 ml eppendorf tube and the volume was adjusted with RNase-free water to 250 µl. 250 µl of Buffer OBB (20 mM Tris·Cl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS, QIAGEN) was added and prewarmed to 37°C. 15 µl of Oligotex Suspension (1 mg/10 µl oligotex particles in 10 mM Tris·Cl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1%

NaN_3) was added and the contents were mixed thoroughly by pipetting up and down the tube. The sample was incubated for 3 min at 70°C in a waterbath and placed at room temperature for 10 min. The Oligotex:mRNA complex was pelleted by centrifugation for 2 min at 14,000 g and the supernatant was removed carefully. The Oligotex:mRNA pellet was resuspended in 400 µl of Wash Buffer OW_2 (10 mM Tris·Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) by votexing and pipeted onto a small spin column placed in a 1.5 ml eppendorf tube, centrifuged for 1 min at 14,000 g. The spin column was transferred to a new RNase-free 1.5 ml eppendorf tube, and 400 µl Buffer OW_2 was applied to column. The column was centrifuged for 1 min at 14,000 g and the flow-through was discarded. The spin column was transferred to a new RNase-free 1.5 ml eppendorf tube, and 30 µl hot (70°C) Buffer OEB (5 mM Tris·Cl, pH 7.5) was pipeted onto the column. The resin was resuspended by pipeting up and down three or four times, and centrifuged for 1 min at 14,000 rpm. About 30 µl eluted portion containing mRNA was collected in eppendorf tube.

2.2.5.3 RT-PCR

1 µg of high quality poly $(A)^+$ RNA together with 500 ng of oligo (dT) primer in a total volume 5 µl was heated to 70°C for 10 min in a thermal cycler (Perkin Elmar 9600/2400) and incubated on ice. The reaction mixture was then made up to 19 µl by adding 4 µl of 5× first strand reaction buffer (provided with the reverse transcriptase), 2 µl of 0.1 M DTT and 8 µl of 1.25 mM dNTP mix and heated to 42°C for 2 min. Reverse transcription was started by the addition of 1 µl of SuperScript II reverse transcriptase (Gibco BRL) and incubated at 42°C for 50 min. After heated to 70°C for 15 min, reaction was made up to 100 µl by the addition of 80 µl sterile water and stored at –20°C. 10 µl of reverse transcription product was used for PCR together with 1× reaction buffer, 200 µM dNTPs, 0.2 µM of For(Balv3) and Rev(Bclstandard) primers, 10% DMSO, sterile water and 1 µl pfu Turbo (STRATAGENE) in a total volume 50 µl. The PCR reaction was performed according to the following program: denaturing at 94°C for 5 min; 35 cycles: denaturing at 94°C for 1 min, annealing at

60°C for 45 sec and elongating at 72°C for 2.5 min. Finally the reaction was incubated at 72°C for 10 min.

2.2.5.4 Digestion and purification of DNA

PCR product was extracted by phenol, precipitated by the addition of 1/10 vol of 3 M Na-Acetate pH 5.2 and 2 vol of ethanol and digested with *Bcl I* and *Bal I*. In the meanwhile, plasmid mCD44v4-10D10-3 was digested with the same restriction enzymes. The digested product was purified by using Easy Pure DNA purification Kit (Biozyme): the digested product was firstly run on TAE agarose gel and the corresponding gel slice was excised out of the gel. The gel slice was weighed and 3 vol of Gel Salt (provided in the Kit) was added according to the weight of gel (100 mg =100 µl) to dissolve the gel at 55°C for 5 min with shaking. Bind (5 µl plus additional volume according to the DNA amount, 1 µl is required for 1 µg DNA) was added, mixed well and incubated at room temperature for 5 min. The beads containing DNA were precipitated by centrifugation at 13,000 g at room temperature for 10 seconds and air-dried. 20 µl sterile water was added, mixed with beads vigorously and incubated at room temperature for 5 min. After centrifugation at 13,000 g for 1 min, supernatant containing DNA was carefully removed.

2.2.5.5 Ligation

In all cases, the insert and vector were loaded on agarose gel to check the DNA content before ligation, and ligated at the amount ratio of about 4:1 (insert:vector). Ligation was performed in a total volume of 20 µl with insert, vector, 1x ligation buffer and 1 µl of T4 ligase (Gibco BRL) and incubated at 16°C overnight. When the vector digested with one restriction enzyme was used for ligation, it was first treated with CIP (Calf intestine phosphatase, Gibco BRL). 25 µl vector (about 2 µg), 3 µl 10× working buffer were mixed with 2 µl of CIP and incubated at 37°C for 1hr before heat inactivation at 55°C for 15 min. 1 µl of CIP was added again and incubated at 37°C for another one hour. Then vector was recovered by Easy Pure DNA purification Kit (Biozyme).

2.2.5.6 Transformation

Basically, DNA was introduced to competent DH5α. In some cases where DNA methylation negative background was required, competent JM110 was used alternatively. 10 µl of plasmid (1 ng/µl) or half of the ligation product was mixed with 100 µl competent bacteria, incubated on ice for 40 min and then heat pulsed at 42°C for 90 sec. DNA was put back on ice for 3-5 min, and 1 ml of LB medium (ampicillin free) was added. After incubation at 37°C with shaking for 1 hr, in the case of plasmid transformation, 100 µl of bacteria were plated on LB agar plate (with 100 µg/ml of ampicillin) and incubated at 37°C overnight. When ligation product was used for transformation, bacteria were precipitated by centrifugation at 6000 rpm for 3 min and all were spreaded on plate.

2.2.6 Site-directed mutagenesis

Mutagenesis was performed according to the instruction manual of QuickChange™Site-Directed Mutagenesis kit (STRATAGENE). The reaction was set up as indicated below: 5 µl of 10× reaction buffer, 50 ng of DNA template (mCD44v8-10-D10-3, prepared from DH5α bacteria), 125 ng of primer C286A-5′ and C286A-3′ respectively, 1 µl of dNTP mix (provided in the kit), ddH_2O was added to make a final volume of 50 µl and 1 µl of pfu DNA polymerase (2.5 µg/µl) was added. The reaction was subject to the following PCR program: segment 1: 95°C, 30 sec; segment 2: 95°C, 30 sec; 55°C, 1 min; 68°C, 10 min; 16 cycles totally. After PCR amplification, 1 µl of the *DpnI* restriction enzyme (10 U/µl, provided in the kit) was directly added to reaction and incubated at 37°C for 1 hr. 10 µl of *DpnI* treated DNA was transferred to 100 µl of Epicurian Coli XL1-Blue supercompetent cells. After heat pulsed at 42°C for 45 sec, the transformation reaction was placed on ice for 2 min. 0.5 ml of LB medium (ampicillin free) was then added and incubated at 37°C for 1 hr with shaking. All the bacteria were plated on LB plate (100 µg/ml of ampicillin) and

incubated overnight at 37°C. Colonies were inoculated for plasmid mini-preparation. Mini-prepared plasmid was directly used for sequencing by using primer P6.

2.2.7 Sequencing

Sequencing was performed by using THERMO Sequenase kit (Amersham LIFE SCIENCE). 4 termination mixes were prepared by using 2 µl of dGTP termination master mix and 0.5 µl of [α-^{33}P] ddNTP for each sequence. Reaction mixture was prepared as the following: 2 µl of reaction buffer, 0.6 µg of DNA, 2 pmol of primer, 2 µl of Thermo Sequenase DNA polymerase, ddH_2O was added up to a final volume of 20 µl. 4.5 µl of reaction mixture was transferred to each termination tube (containing 2.5 µl termination mixes), and PCR reaction was performed as follows: 95°C, 30 sec; 55°C, 30 sec; 72°C, 1 min, 30 cycles. Samples were heated to 72°C for 5 min immediately before loaded on sequencing gel.

2.2.8 Cell culture

All cells were maintained at 37°C in an incubator (Forma Scientific, Labotect GmbH, Göttingen) in 5% CO_2 and 95% air humidity. All cells were grown in petri dishes (Greiner Labortechnik, Flikenhausen) of varing sizes depending on the application. Storage of cells were done by harvesting cells and adding 1 ml of freezing medium (10% DMSO (Fluka Chemie AG. Switzerland) in FCS). The cells were transferred to cryovial, left on ice for 1hr and transferred to –80°C for 1 day before finally to liquid nitrogen. To thaw cells, the cryovial was removed from liquid nitrogen and placed at 37°C for 5-10 min. The cells were transferred to 10 ml of pre-warmed fresh medium followed by light centrifugation before being replated on petri dishes in fresh medium.

2.2.9 Transfection of LB cell line

LB cells were seeded at 0.5-1$\times 10^6$/ml one day before transfection. On second day, cells were washed with complete LB media twice, resuspended in 0.4 ml complete LB

media and transferred to electroporation cuvette (1×10^7 cells /0.4 ml in one cuvette, BIORAD). 40 µl DNA (20 µg in PBS w/o Ca/Mg) was mixed into media and incubated on ice for 10 min. Electroporation was fulfilled at 220 v, 960 µF by using Gene Pulser™ (BIORAD), and immediately after electroporation 0.4 ml ice-cold FCS was added. The cells were left on ice for 5 min, and then transferred to LB medium. For transient transfection, cells were harvested 20 hr after passage and measured by using individual methods. For stable transfection, old medium was replaced with selective medium 2 days later.

2.2.10 Development of Tet-off LB cell line

2.2.10.1 Outline of establishing Tet-off LB cell line

The principle of Tet-off system is illustrated in Fig. 4.

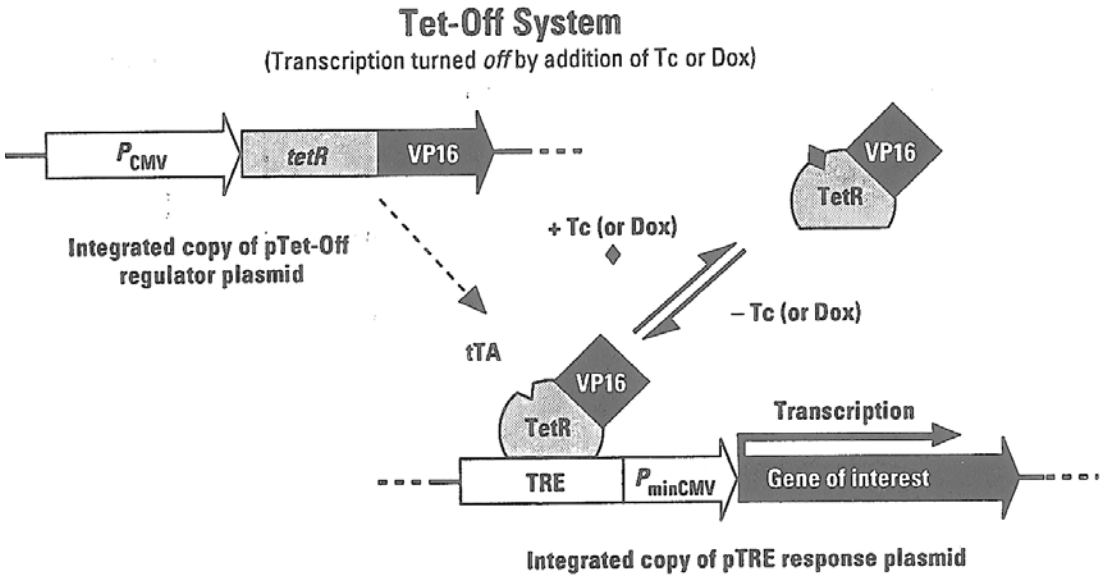


Figure 4. Schematic representation of gene regulation in the Tet-off system. The tetracycline-controlled transactivator (tTA) is a fusion of the wildtype Tet repressor (TetR) to the VP16 activation domain (AD) of herpes simplex virus. The tet-responsive element (TRE) consists of seven copies of the 42-bp tet operator sequence (tetO). The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (P_{minCMV}), which is silent in the absence of activation. tTA binds the TRE-and thereby activates transcription of Gene X-in the absence of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox).

To establish conditional expression of CD44 in LB cells, two steps of stable transfections are required (Fig. 5).

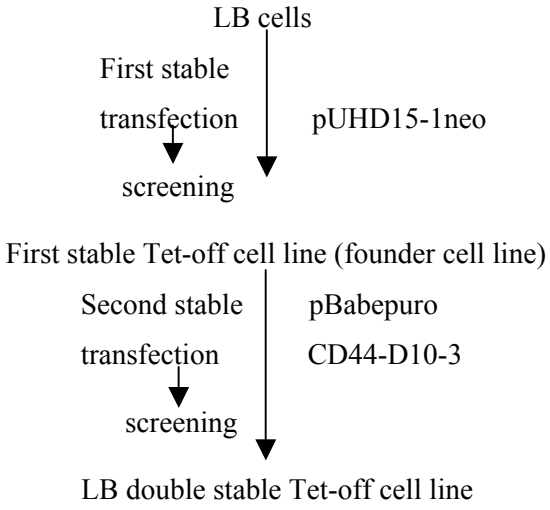


Figure 5. Overview of developing Tet-off and double-stable Tet-off cell lines

In the first stable transfection, A plasmid which harbors tTA (pUHD15-1 neo) is transfected and stable clones are screened by transiently expressing a plasmid which contains luciferase reporter gene under the control of tet-responsive element (TRE) and minimal CMV promoter (Pmin CMV) (pUHC13-3) in the absence or presence of Dox. The criteria for selecting clones is to find clones with the highest induction of exogenous gene expression by removing Dox and the lowest exogenous gene expression in the prescence of Dox. In the second stable transfection, a plasmid containing CD44 under the control of TRE and Pmin CMV (CD44-D10-3) will be introduced to the first stable Tet-off cell line together with a puromycin-encoding plasmid (pBabepuro). The clones are screened by FACS analysis for CD44 expression.

2.2.10.2 Transfection and selection of stable cell lines

In the first transfection, pUHD15-1neo was introduced into LB cells. Clones were obtained by limiting dilution, namely, 2 days after transfection, cells were counted and seeded in a 96-well flat-bottom microtiter plate at 2×10^4 cells/well/200 µl medium with 200 µg/ml of G418 Usually, after two weeks of incubation, resistent clones were

visible and then transferred to 24-well plate and expanded in 6-well plate subsequently.

2.2.10.3 Screening stable cell lines

Transient transfection assays were performed to identify good clones. About 5×10^6 cells were transfected with 5 μg pUHC13-3 and transfected cells were split into 2 wells of 6-well plate, one well with 1 μg/ml of Dox in LB medium, while the other without. 16 hr later, cells were subject to luciferase assay: cells were washed with PBS(w/oCa/Mg) at 4°C twice and lysed with 1× Passive Lysis buffer (PROMEGA). The lysates were kept on ice for 15 min, mixed occasionally in between, and cleared by centrifugation at 13,000 g for 5 min at 4°C. 50 μl lysate was transferred to 96-well plate (LIA-plate, white, flat-bottom, medium binding, Greiner Labortechnik), mixed with 150 μl luciferase assay solution (for 10 samples: 1379 μl of Glycylglycine buffer (25 mM Glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA), 1 μl of 1 M DTT, 20 μl of 100 mM ATP, 100 μl of luciferin stocking solution (1 mM luciferin (potassium salt from beetle, Promega) in Glycylglycine buffer)) by automatic injection of MICROBETA Jet (1450, WALLAC) and luciferase activity was measured according to instruction manual of MICROBETA Jet.

Once the good clones were achieved, aliquots were frozen with freezing medium (10% DMSO in FCS). Transient expression of CD44 was carried out in the similar way to that of luciferase and CD44 expression was detected by FACS staining.

2.2.10.4 Development of double-stable Tet-off LB cell line

The first stable transfectants were transfected with 20 μg of mCD44-D10-3 and 1 μg of pBabepuro. After electroporation, 1 μg/ml Dox was added to culture. 2 days later, individual clones were selected against 1 μg/ml of puromycin and 200 μg/ml of G418 in LB medium with 1 μg/ml of Dox by limiting dilution again and screened by FACS analysis of CD44 expression in the presence and absence of Dox.

2.2.10.5 Culture of double-stable tet-off cell lines

Usually, transfectants were maintained in LB medium with 100 μg/ml G418 and 10 ng/ml of Dox. CD44 expression was completely switched on by washing cells with PBS (w/oCa/Mg) containing 3% FCS for three times on the first day, and another three times on the second day.

2.2.11 Flow cytometry

2.2.11.1 CD44 expression

Cells were harvested and washed with FACS staining buffer (3% FCS and 0.1% NaN_3 in PBS w/oCa/Mg) twice at 4°C. Cells were transferred to 96-well plate at 0.5×10^6 cells/well/sample, and centrifuged at 1400 rpm for 2 min at 4°C. Supernatant was poured off. First mAb was added to cell pellet and incubated at 4°C for 45 min. Cells were washed twice, and incubated with FITC- or PE- labelled secondary Ab at 4°C for 45 min. In the case of direct staining, cells were incubated with labelled mAb at 4°C for 45 min. After washed twice cells were subject to FACS Star plus flow cytometer (Becton Dickinson).

2.2.11.2 HA binding

Cells were harvested and washed with FACS staining buffer. About $0.5\text{-}1\times10^6$ cells were aliquoted to each well of 96-well plate and precipitated down by centrifugation at 4°C for 2 min. The cell pellet was resuspended with 50 μl of FITC-HA (1:100 in FACS staining buffer) and incubated on ice for 45 min. After staining, the cells were washed and the fluorescence was analyzed by FACS-Star plus flow cytometer. When KM 8.1 mAb was used to block CD44-dependent HA binding, 50 μl of KM8.1 (1.7 μg/μl, 1:50 dilution in FACS staining buffer) was incubated with cells on ice for 30 min before the addition of HA-FITC.

2.2.11.3 Two-color flow cytometry

Cells were first stained with FITC-HA for 45 min and washed once. Cells were incubated with anti-CD44 mAb for 45 min on ice, and subsequently with second Ab as described above.

2.2.12 Pulse-labelling of LB cells

2×10^7 cells were harvested and washed with 6 ml prewarmed pulse-labelling medium (10% dialyzed FBS (GIBCO BRL), 25 mM HEPES in RPMI (w/o Met, Cys, L-Glu, SIGMA)) twice. Cells were resuspended in 4 ml of pulse-labelling medium and incubated at cell culture incubator. After 15 min, cells were then harvested, resuspended in 2 ml of pulse-labelling medium containing cell labelling Pro-mix [^{35}S] (Amersham Life Science) (0.5 mCi/ml) and labelled in a special incubator (for radioactivity work) for another 4 hr. The labelled cells were harvested, washed with ice-cold PBS at 4°C for three times and lysed in lysis buffer as used in immunoprecipitation. The CD44 was immunoprecipitated in the normal way and fractionated by SDS-PAGE. The gel was fixed with fixation solution (isoproponal:acetic acid:H_2O =25:10:65) for 30 min, amplified with Amplify (Amersham Pharmacia Biotech) for 20 min, dried under vacum for 2 hr, and exposed to X-ray film.

2.2.13 Cell biotinylation

LB cells were harvested and washed with 4 ml PBS twice. 2.5×10^7 cells were resuspended in 0.5 ml PBS (w/oCa/Mg). 0.5 ml of NHS-LC-Biotin (1 mg/0.5 ml in PBS, PIERCE) was added, mixed well and incubated at room temperature for 30 min (gentlely mixed every 10 min). After labelling, LB cells were washed with PBS twice.

2.2.14 Immunoprecipitation

2.2.14.1 Preparation of antibody-conjugated beads

30 µl of 50% protein G-sepharose beads slurry (Oncogene) together with 5 µg IM7 and 500 µl ice-cold PBS (w/oCa/Mg) were tumbled end over end at 4ºC for ≥4 hr in a

tube rotator. The beads containing IM7 were precipitated by centrifugation at 4ºC for 10 sec. After aspiration of the supernatant the beads were washed with 1 ml of RIPA denaturing lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris·Cl, pH 7.5) twice.

2.2.14.2 Cell lysis and pre-clearing

LB cells: for general Western blot, cells were harvested, washed with PBS and directly lysed with 2× non-reducing buffer (125 mM Tris·Cl, pH 6.8, 4.1% SDS, 17.4% glycerol, 10 mM iodoacetamide (SIGMA), 1 mg/100 ml bromphenol blue). To shear DNA, cells were passed through 26G syringe needle. The cell lysate was denatured at 95°C for 5 min before loading on SDS-PAGE. For detecting biotinylated CD44 and ^{35}S-labelled CD44, labelled cells were lysed with 1× RIPA. 1 ml of RIPA denaturing lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris·Cl, pH 7.5, 1 mM PMSF, 1x Complete™ Mini protease inhibitor cocktail, BOEHRINGER MANNHEIM) was added to the cell pellet (about 2.5× 10^7 cells) and the cells were resuspended by pippeting up and down for several times. After 15-30 min of incubation on ice, the lysate was cleared by centrifugation at 13,000 g for 15 min at 4ºC. The supernatant was then transferred to another eppendorf tube containing 30 µl of 50% protein G-sepharose beads slurry, tumbled end over end at 4ºC for 30 min. The supernatant was recovered by centrifugation at 13,000 g at 4ºC for 5 min.

2.2.14.3 Immunoprecipitation

10 µl 10% BSA was mixed with the above antibody-conjugated beads and transferred to this tube the precleared cell lysate. The content was incubated overnight while mixing end over end in a tube rotator at 4ºC. The beads were precipitated by centrifugation at 13,000 g at 4ºC for 10 sec and washed with 1 ml of ice-cold washing buffer (0.1% NP-40, 150 mM NaCl, 50 mM Tris·Cl pH 7.5, 1mM PMSF) for 4 times and ice-cold PBS once. The samples were kept on ice for 5 min after each wash and the total wash time should be about 30 min. The immunoprecipitated product (from

2.5× 10^7 cells) was dissolved in 400 µl 2× non-reducing buffer by shaking at 1400 rpm for 20 min at 68ºC and denatured at 94ºC for 3 min.

2.2.14.4 Western blotting

Proteins resolved by SDS-PAGE were transferred to Immunobilon-PVDF membrane (Millipore) using BioRad Transfer chambers containing transfer buffer (24 mM Tris base, 193 mM glycine, and 10% methanol). Transfer was performed at 4°C overnight.

2.2.14.5 Probing

For the CD44 detection by anti-CD44 mAb: the membrane was blocked for 1 hr in blocking buffer (PBS with 0.3% Tween 20 and 10% dry milk) and incubated with primary antibody in blocking buffer for 1 hr. After 4 times of washing with 0.3% Tween 20 in PBS, the membrane was incubated with HRP labelled secondary antibody (1:1500) at room temperature for 1 hr and washed again with 0.3% Tween 20 in PBS.

For the detection of biotinylated CD44: the membrane was blocked in special blocking buffer (50 mM Tris·Cl, pH7.5, 150 mM NaCl, 0.4% Tween-20, and 5% BSA) for 2.5 hr at room temperature, and incubated with NeutrAvidin™-HRP conjugated (PIERCE) (1 µg/ml) for 1h. After wash, the protein was visualized by ECL reaction (Amersham Life Science) and exposed to Hyperfilm-ECL (Amersham Life Science).

2.2.15 CPC precipitation

LB cells were harvested and washed with PBS for 3 times. Cells were lysed in ice-cold PBS (w/oCa/Mg) containing 0.5% NP-40 and 1 mM PMSF and incubated on ice for 30 min. Insoluble material was removed by centrifugation at 13,000 g for 10 min at 4°C. 100 µl aliquots of the lysate supernatant (about 10^6 cells) were mixed with 50 µl of HA (1 µg/µl) and incubated at room temperature for 1 hr. 350 µl of 1.43% CPC was added, mixed well and incubated at room temperature for 1 hr. The pellets were precipitated by centrifugation at 13,000 g at room temperature for 10 min and rinsed

three times with 1% CPC contaning 30 mM NaCl. The pellets were disolved in 50 µl 2× non-reducing sample buffer. CD44 was fractionated by SDS-PAGE and detected by probing with IM7.

2.2.16 V8 peptide mapping

Cells were metabolically labelled with ^{35}S-methionine or labelled with Biotin. Immunoprecipitated proteins were resolved by SDS-PAGE. The individual gel slices containing CD44 monomer and dimer were excised out of the gel and then soaked for 30 min with occasional swirling in 5 ml of solution A (125 mM Tris·Cl, pH 6.8, 0.1% SDS, and 1 mM EDTA). In the case that CD44 dimer needed to be reduced, 0.5% DTT was included in solution A and gel slice was treated for 1 hr. The gel slices were pushed with a spatula into the bottom of a well of the second gel with stacking gel longer than usual (up to 5 cm). Spaces around the slices were filled by overlaying each gel slice with 10 µl of solution A containing 20% glycerol. Finally 10 µl of solution A containing 10% glycerol and a given amount of v8 protease (SIGMA) were overlayed into each slot and electrophoresis was performed in the normal way except that the current was turned off for 30 min when the bromphenol blue dye neared the bottom of the stacking gel. The gel was fixed with fixation solution (isoproponal:acetic acid:H_2O =25:10:65) for 30 min, amplified with Amplify (Amersham Pharmacia Biotech) for 20 min, dried under vacum for 2 hr, and exposed to X-ray film. When biotinylated CD44 was used for the experiment, CD44 was transferred to PVDF membrane and detected with NeutriAvidin-HRP.

2.2.17 Two-dimensional gel analysis

The first dimension was run in tube gels and the second in slab gels. 20 µl immunoprecipitated CD44 from biotinylated LB cells were loaded on the top of stacking gel and run using the same parameters as common SDS-PAGE. The tube gels were removed carefully from the tube by using a syringe and incubated with slow shaking in 15 ml reducing (0.5% DTT, 125 mM Tris·Cl, pH 6.8, 0.1% SDS) or non-reducing (omitting DTT) buffer at room temperature for 1 hr. The preparation of the

second-dimension gel is normal with the exception that without inserting the comb into the stacking gel so as to achieve an even stacking gel surface for tube gels. After layed carefully on the stacking gel surface of the second-dimension gel, the tube gels were affixed using 1% agarose containing 125 mM Tris·Cl, pH 6.8 and 2% SDS. The proteins were fractionated, transferred and probed as before.

2.2.18 *In vivo* tumor growth and metastasis

BALB/c mice and MF1 nude mice were kept under standard pathogen-free conditions. Cultured cells (mycoplasma free) were harvested, washed with PBS (w/oCa, Mg) twice and resuspended in PBS containing 1 μg/ml Dox. $1\text{-}3\times10^6$ cells (in 0.1 ml PBS) were injected subcutaneously into the left flank of mouse. After injection, mice were divided into two groups, one fed with normal water and the other with Dox (100 μg/ml) in drinking water. 8 Days post injection, tumor size was measured by calipters and thereafter, tumor size was recorded every 2-3 days. Tumor volume was calculated according to the following formula: $\text{volume}=\text{length}\times\text{width}^2/2$. Approximately two weeks later, mice were sacrificed and dissected. The matastasis formation was checked by isolating the invaded cells from lymph node or spleen and putting cells back to culture medium containing selection antibiotics. Live cells were checked by microscope and in some cases, by FACS staining.

PART THREE

RESULTS

3.1 Establishment of regulated expression of CD44 by Tet-off system

To study the roles of CD44 variant isoforms in lymphoma development, we decided to make use of the Tet-off gene expression system to establish LB lymphoma cell lines in which CD44 expression can be conditionally modulated. To generate such cell lines, firstly, a plasmid containing tetracycline-controled transactivator (tTA) (pUHD15-1neo) has to be introduced into LB cells. To achieve conditional expression, different CD44 constructs harboring CD44 isoforms under the control of tet-responsive element (TRE) and CMV minimal promoter are subsequently transfected to LB cells that are constitutively expressing tTA.

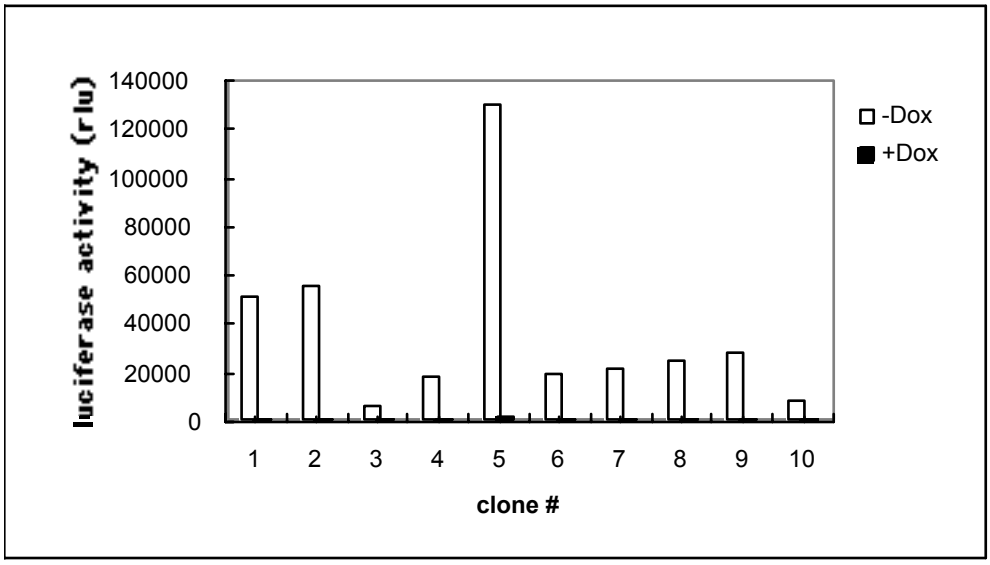
3.1.1 Establishment of stable transfectants conditionally expressing CD44s, CD44v3-10, CD44v4-10 and CD44v8-10

At first, we determined the concenration of Dox at which the transfected gene could be induced by using a Tet-off control cell line in which luciferase reporter gene is regulated by Tet-off system (CHO-AA8-Luc). 1 μg/ml of Dox was found to be enough to get full activation and inactivation of gene expression and was used in all of the following experiments. Plasmid containing tetracycline-controled transactivator (tTA) was transfected into LB cells and 150 clones were picked. To identify the clones that have the highest induction of exogenous gene expression in the abscence of Dox and the lowest exogenous gene expression in the prescence of Dox, we screened these 150 clones by transient transfection of a plasmid containing the luciferase gene under the control of tet-responsive element (TRE) and the CMV minimal promoter (PUHC13-3). Ten clones demonstrated nice induction of luciferase reporter gene without Dox in culture medium and background luciferase activity in the presence of Dox (Fig. 6A). Clone 1 (referred to as founder cell line afterwards)

shows the highest induction (67 fold) and the lowest background and therefore was chosen for the second transfection (Fig. 6B).

To test such an induction works also for CD44 expression we used one CD44 construct (CD44v4-10-D10-3, see 2.1.3.2) harboring CD44v4-10 isoform under the control of TRE and CMV minimal promoter in transient transfection into the tTA-expressing founder cell line. To monitor CD44 expression immunofluorescence staining was used. Since the staining dye also reacted with the dead cells that occurred upon transfection we made double transfections with CD44v4-10 construct and a plasmid containing GFP gene under the control of CMV promoter (pEGFP-N1). GFP expression monitors live cells and double positive cells (shown in the upper right region of each figure in Fig. 7) are live cells expressing both CD44 and GFP. Such double positive cells can not be detected in control staining and CD44 staining of the cells with Dox, but can be measured in CD44 staining of the cells in the abscence of Dox. In anti-pan CD44 staining, double positive cells are present in the prescence of Dox because of the endogenous CD44 expression.

A.



B.

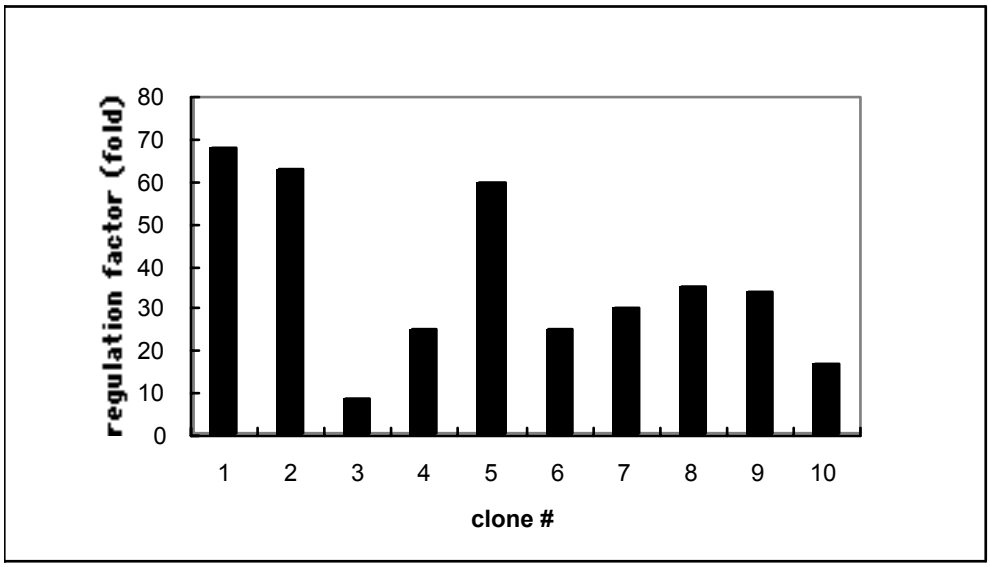


Figure 6. A. Regulation of luciferase activity of the first stable Tet-off clones by Dox. Cells from 10 respective clones were transfected with luciferase reporter plasmid (pUHC13-3), split equally into six-well plate and treated with or without Dox. After 12 hr, cells were harvested and luciferase activity was measured. **B. Regulation factors of LB first stable Tet-off clone.** The regulation factor was calculated as the ratio of luciferase activity of the cells cultured without Dox to that of cells cultured with Dox.

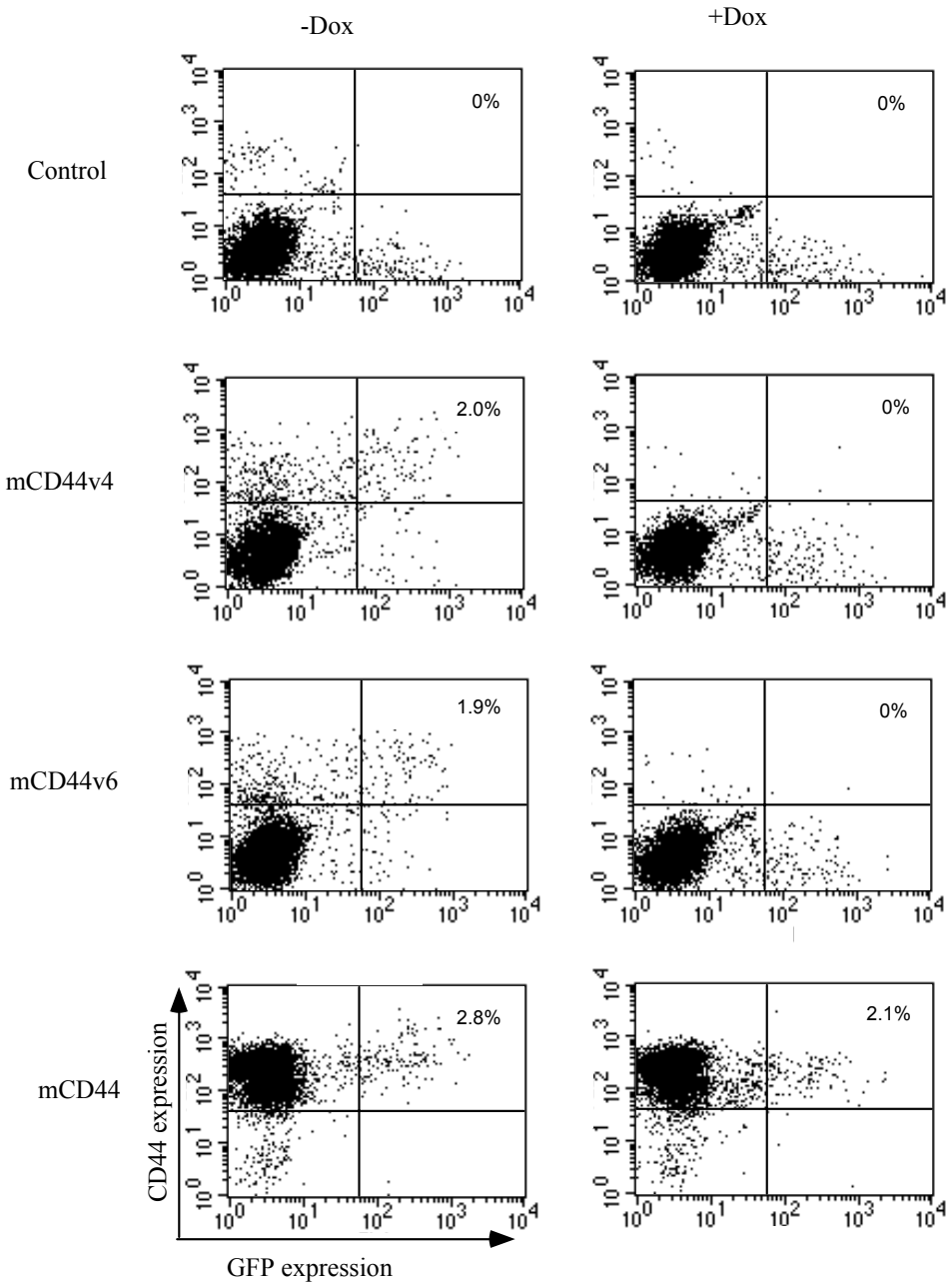


Figure 7. Transient expression of mCD44v4-10 is regulated by Dox in LB founder cell line (clone #1). Cells were cotransfected with mCD44v4-10-D10-3 and pEGFP-N1 at the ratio of 20:1, split into six-well plate and cultured with or without Dox. After 48 hr, cells were harvested and stained with control Ab (Rat IgG), anti-CD44 v4, v6 or pan-CD44 mAbs for 45 min. After wash, cells were stained with PE-lablled secondary Ab (Goat anti-Rat Ig (H+L), mouse absorbed, PharMingen) for 45 min and subjected to FACS star plus flow cytometer (Becton Dickinson). CD44 and GFP expression were analysed by gating out live cells in flow cytometric analysis.

The expressions of CD44 variant isoforms are upregulated in tumorigenesis and metastasis. For instance, CD44 v6 and CD44 v7/8 expressions were found to be related to the progression of human colorectal tumor and cervical carcinoma respectively (Naor et al., 1997). To study the roles of CD44 variant isoforms in lymphoma development, we made different CD44 constructs harboring CD44v3-10, CD44v4-10, CD44v8-10 and CD44s under the control of tet-responsive element (TRE) and CMV minimal promoter (Fig. 2). The above transient expression experiment proved that CD44 could be expressed and regulated by Dox in LB cell line, therefore, we stably introduced these constructs to the tTA-expressing founder cell line subsequently. 2% of the picked clones have the high exogenous CD44 expression in the abscence of Dox and little expression in the prescence of Dox. For each CD44 isoform transfection, we isolated three such clones for further study. Fig. 8 presents the CD44 expression of the representative clones from each transfectant. Expression of CD44 variant isoforms reach high level upon the withdrawl of Dox and their expression can be downregulated to the basal level by the addition of Dox. The effect of Dox is CD44 specific, since no regulation of CD8 by Dox was observed in these clones (data not shown). The relative fold induction of CD44s transfectant is lower due to the endogeneous CD44s expression. CD44 expression was also checked by Western blot (Fig. 9). The migration pattern of CD44 as seen on Western blot is due to complex posttranslational modification (mostly glycosylation).

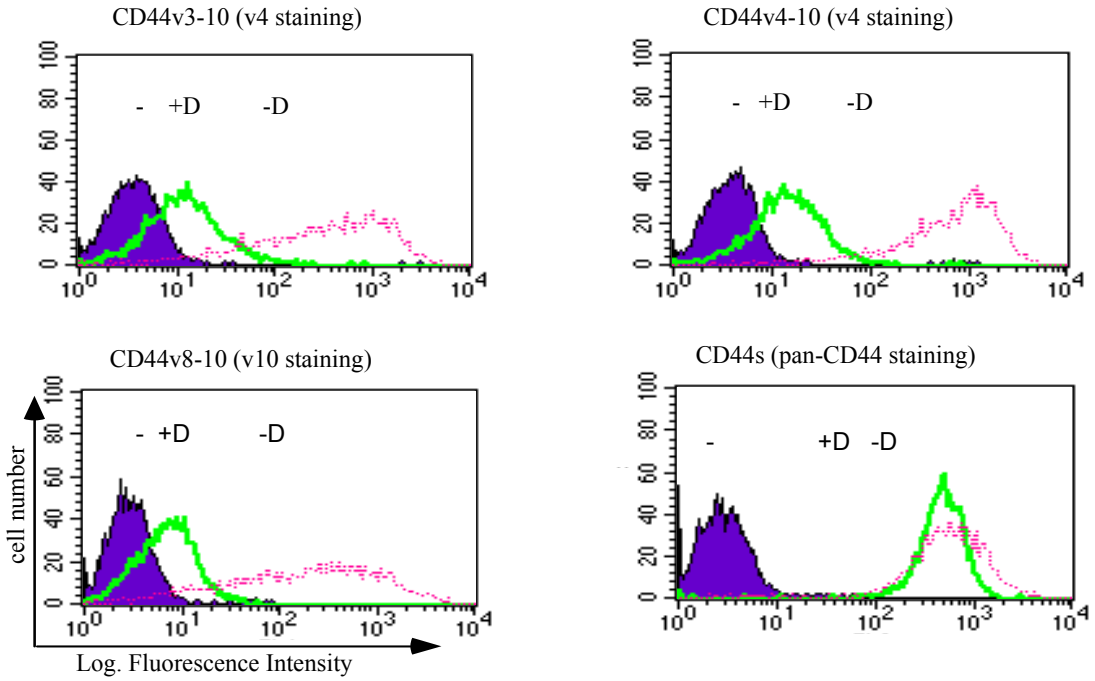


Figure 8. Regulation of CD44 expression by Dox (D). One representative clone from CD44v3-10, CD44v4-10, CD44v8-10 and CD44s transfectants was used for the analysis. Dox was eliminated from cellls by intensive washing of cells and the cells were allowed in culture for 2 days. The cells cultured with or without Dox were harvested, stained with different anti-CD44 mAbs (CD44v3-10, v4-10 transfectants were stained with anti-v4 mAb, CD44v8-10 with anti-v10, and CD44s with anti-pan CD44 mAb) and secondary Ab. CD44 expression was analysed by flow cytometry.

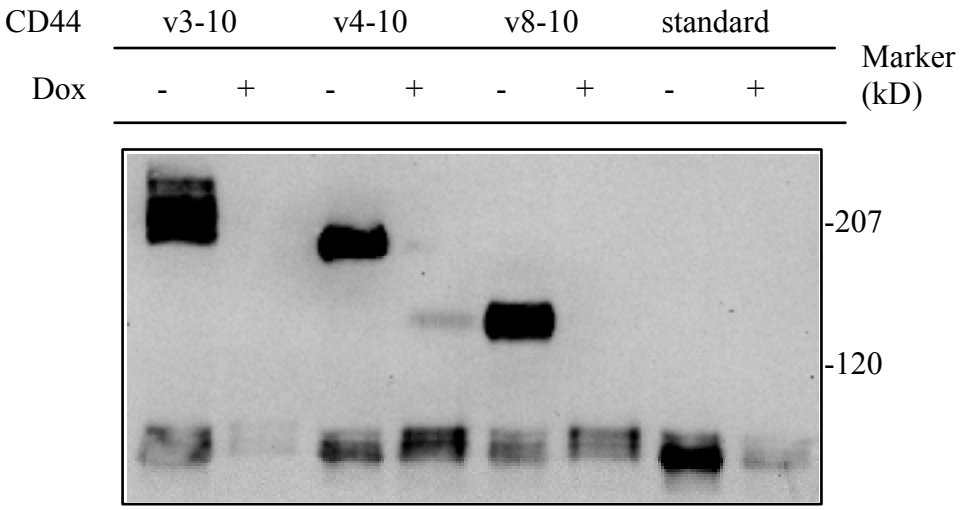


Figure 9. Western blot analysis of conditional expression of CD44 isoforms. The transfectants cultured with or without Dox were lysed with reducing lysis buffer (125 mM Tris·Cl, pH6.8, 4.1% SDS, 17.4% glycerol, 10 mM iodoacetamide, and 1 mg/100ml bromphenol blue, 0.15% DTT) and loaded on 7.5% SDS-PAGE gels. After transfer to PVDF membrane, CD44 was probed with anti-pan CD44 mAb (IM7).

3.1.2 Characterization of CD44 induction

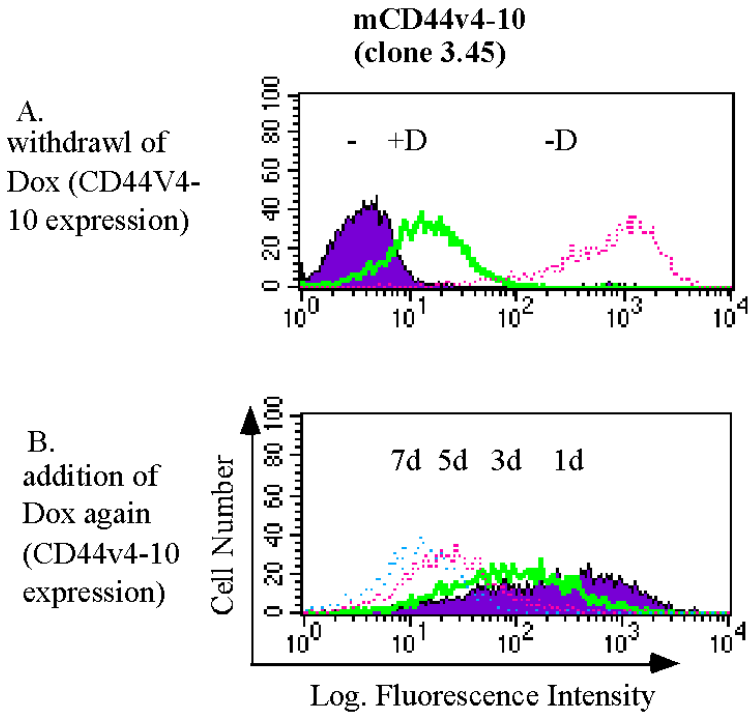


Figure 10. Time kinetics of conditional CD44 expression. A. CD44v4-10 transfectant (clone 3.45) was used. Dox (D) was withdrawn from medium and two days later, the CD44 expression was measured by staining the cells with anti-CD44v4 mAb. B. The cells expressing high-level CD44v4-10 were treated with Dox by the addition of Dox back to culture medium for different time period. Finally, treated cells were harvested and CD44 expression was measured by FACS analysis.

We also characterized the time kinetics of keeping CD44 expression on and off. The LB double stable Tet-off cell lines were usually cultured with Dox to avoid ectopic CD44 expression. For induction of CD44 expression, Dox was removed by intensive washings and complete CD44 expression was obtained on third day (Fig. 10A). CD44 expression was gradually downregulated by Dox (Fig. 10B) and the entire turnoff took seven days.

3.2 Ligand binding properties of LB transfectants

CD44 is a family of glycoproteins present on many cell types. One of the ligands for CD44 is HA which is a major ECM component. The binding of CD44 isoforms to HA is known to cause cell adhesion to ECM and is implicated in the stimulation of cell proliferation and cell migration (Bourguignon et al., 2000; Bourguignon et al., 1998).The HA binding property of CD44 has also been correlated with tumorigenesis and metastasis at least in certain cell types (Naor et al., 1997). For instance, the interaction between CD44s and HA seems to be involved in melanoma tumor formation (Bartolazzi et al., 1994). In a murine mammary carcinoma cell line which expresses several types of CD44 isoforms, expression of soluble CD44 abolished the HA binding of tumor cells as well as the tumor growth and metastasis (Yu et al., 1997). The binding of CD44 to HA is influenced by a variety of factors, for instance, CD44 isoforms expressed and post-translational modifications of CD44 molecules. We therefore analysed the HA-binding capacity of the different CD44 isoforms in LB lymphoma cells.

3.2.1 All CD44 isoforms enhance HA binding ability of LB cells

LB transfectants expressing only endogenous CD44s (in the prescence of Dox) show very low HA binding capacity as revealed by FACS analysis using fluorescein-labeled HA (FITC-HA) (Fig. 11). Upon induction of CD44 expression, the HA binding of all transfectants, no matter whether they express CD44v4-10, v3-10, v8-10 or CD44s, was dramatically increased. This CD44-mediated HA binding is specific because it could be blocked by KM81 mAbs which bind to the HA binding site of CD44 (Fig. 11) and could be competed with unlabeled HA (data not shown).

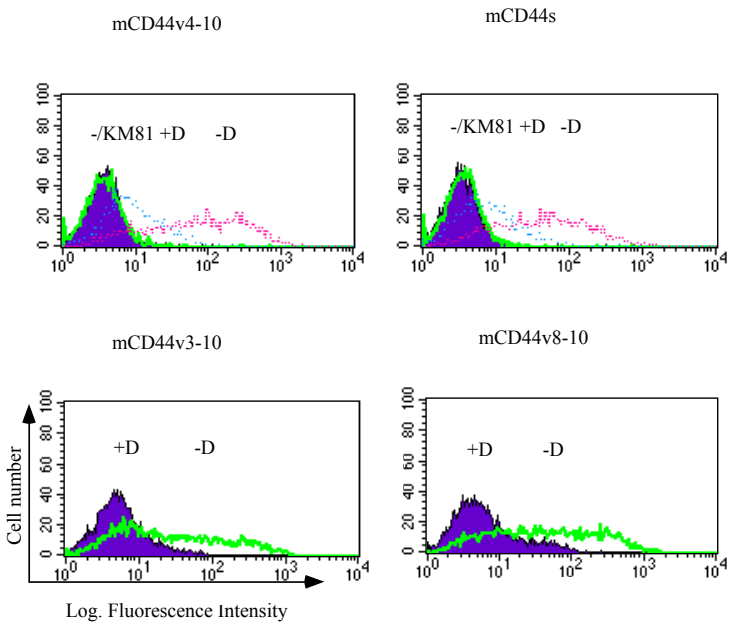


Figure 11. Regulation of HA binding by Dox. The CD44v4-10-, CD44v3-10-, CD44v8-10- and CD44s-transfectants were cultured for two days with or without Dox and harvested. HA binding was measured by staining the cells with FITC-HA and analysed by FACS. In the antibody (KM81) blocking experiment, the cells were first incubated with KM81 and then stained with FITC-HA.

3.2.2 Four CD44 isoforms have the similar HA binding capacity

In previous experiment, HA binding was measured at full induction of ectopic CD44 expression. In a refined analysis we tried to correlate HA binding of different CD44 isoforms with increasing amounts of CD44 expressed on the various transfectants.

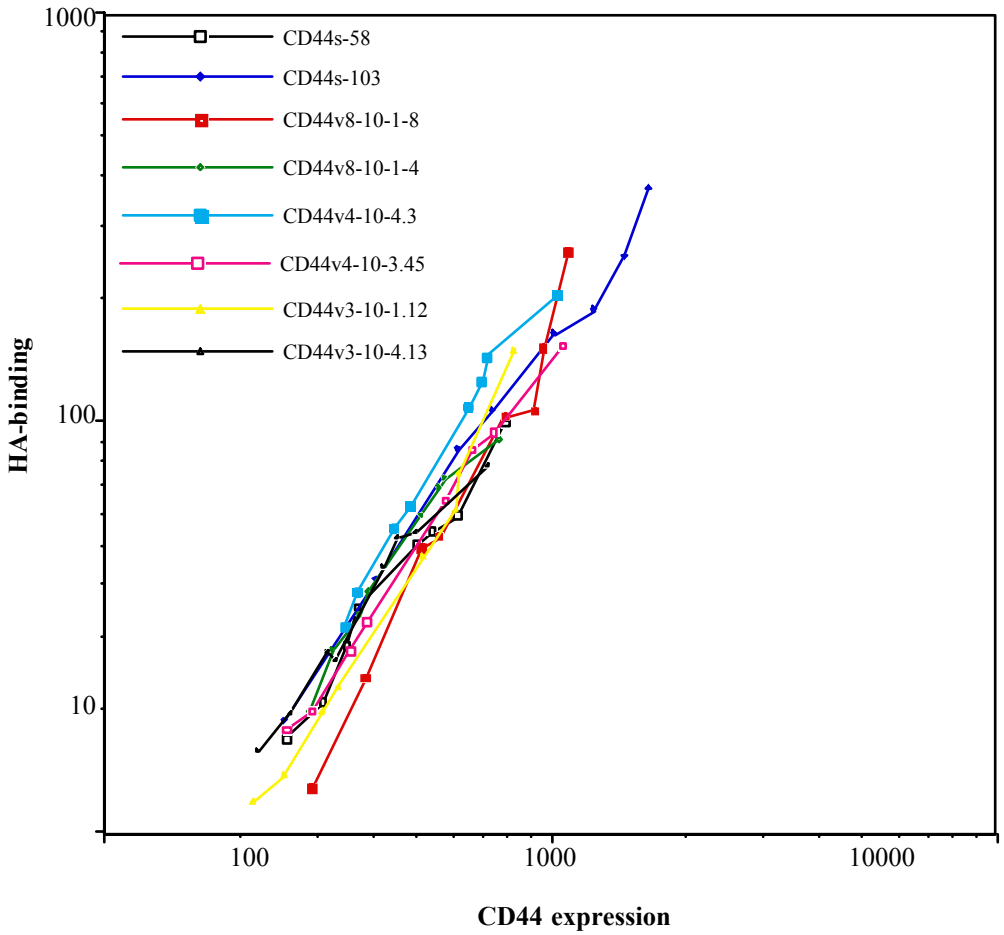


Figure 12. No difference regarding HA binding between cells transfected with CD44s, v8-10, v4-10 and v3-10. Two clones from each transfectant were analysed. CD44 expression was first completely switched on, and the cells were then treated with Dox. After 12, 17, 24, 36, 42 and 68 hr respectively, the cells were harvested and pan-CD44 expression and HA binding were measured by FACS.

For that purpose, we examined clones induced for CD44 expression at different time after stop of CD44 expression by Dox treatment. CD44 expression was firstly completely switched on (by omitting Dox) and then the cells were treated with Dox to suppress CD44 expression. Because of the slow turnover of CD44 on cell surface (Lokeshwar and Bourguignon, 1991), different CD44 amounts were obtained at different time after Dox treatment over a period of several days. After different time, the amounts of CD44 expression and the HA binding capacity were determined (Fig. 12). Two clones each of CD44s-, CD44v8-10-, CD44v4-10- and CD44v3-10-transfectants were used for the following experiments to exclude that the results could be hampered by clonal variation. HA binding increased with increasing amounts of

CD44. This increase was similar for the two clones of each transfection (that excludes clonal influences) and was also similar for all clones regardless which CD44 isoform was expressed. When specific CD44 variant expression was measured by means of an anti-CD44 v10 specific antibody that recognises all variant isoforms used here there was also no difference obserced between the expression level of CD44v and HA binding (Fig. 13).

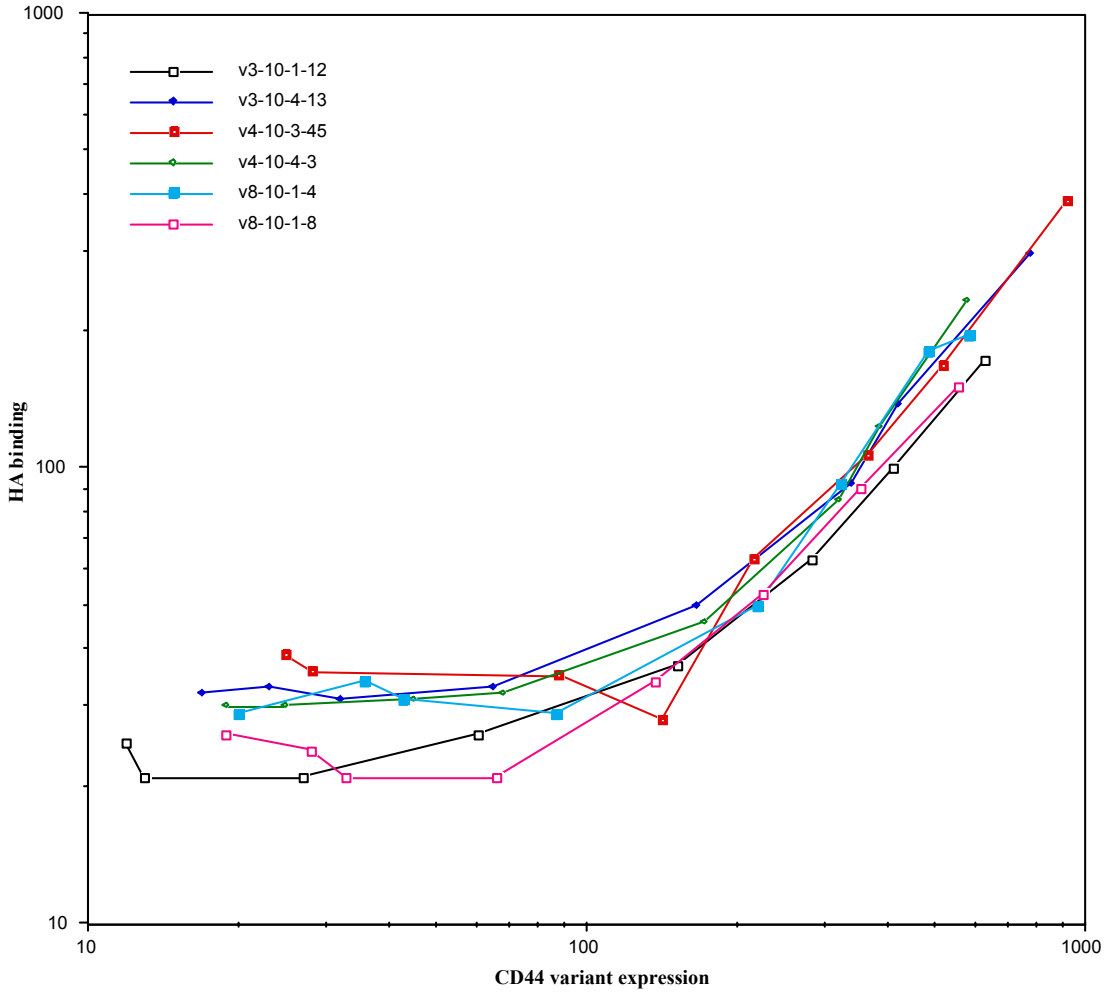


Figure 13. No difference regarding HA binding between cells transfected with v8-10, v4-10 and v3-10. Two clones from each transfectant were analysed. CD44 expression was completely switched on, and the cells were treated with Dox for 0, 0.5, 1, 2, 3, 4, 5, and 6 days. Finally, cells were harvested and CD44 variant expression (anti-CD44 v10 mAb) and HA binding were measured by FACS.

3.2.3 CD44 isoforms bind differentially to other GAG ligands

Besides HA, CD44 binds to other glycosaminoglycans (GAGs) and thereby is able to associate with proteoglycans such as aggrecan and versican (Fujimoto et al., 2001;

Kawashima et al., 2000). In a rat tumor system, CD44v6-7 isoform can bind a number of GAG ligands specifically HA, chondroitin sulfate (CS) A, B and C, heparin (H) and heparan sulfate (HS) and thus expand the repertoire of ligand binding (Sleeman et al., 1997). To answer whether CD44 isoforms expressed in LB cells can bind other GAGs, we used the CPC precipitation approach. The rationale of this method is that GAGs can be precipitated by cationic detergents such as CPC due to salt formation. If CD44 in the cell lysate binds to GAGs, then it coprecipitates with CPC. In the CPC precipitates the CD44 isoforms can be identified by Western blot analysis using CD44 specific antibodies. Interestingly, we found that the binding of CD44s to HA differed from CD44v isoforms in CPC experiment (Fig. 14) in contrast to the FACS experiment (Fig. 12). Mock-transfected cells that express only endogenous CD44s demonstrated no HA binding. Upon overexpression of CD44s in LB cells, these transfectants could bind to HA, but the comparison with CD44v transfectants revealed that CD44s bound to HA to a much less extent than CD44v isoforms. There is also a sharp difference between CD44s and CD44v regarding the binding to other GAG ligands. CD44s did not bind to either of them, whereas expression of CD44v3-10, CD44v4-10 and CD44v8-10 led to strong binding to CSA, B and C, and also, although weaker, to H, HS and keratan sulfate (KS). Also among the CD44 variant isoforms there seem to be a difference regarding their GAG binding. The binding ability of CD44v3-10 seems to be highest while that of CD44v4-10 and CD44v8-10 is similar.

In conclusion, LB cells achieve similar HA binding ability after transfection with CD44v8-10, CD44v4-10 and CD44v3-10 as measured by FACS analysis. These CD44 isoforms can also bind to other GAG ligands in CPC precipitation and CD44v3-10 displays the highest binding ability. With regard to CD44s, CD44s-transfectants bind HA with similar ability to that of three CD44 variant isoforms in FACS analysis, however, in CPC experiment, CD44s can only bind poorly to HA but not to other GAGs. This apparent contradiction could be due to the different methods used in which CD44 molecules may adopt different structures. FACS analysis measures the binding of CD44 proteins on intact cells to HA, whereas the CPC method reflects the binding of CD44 molecules in cell lysate to ligand.

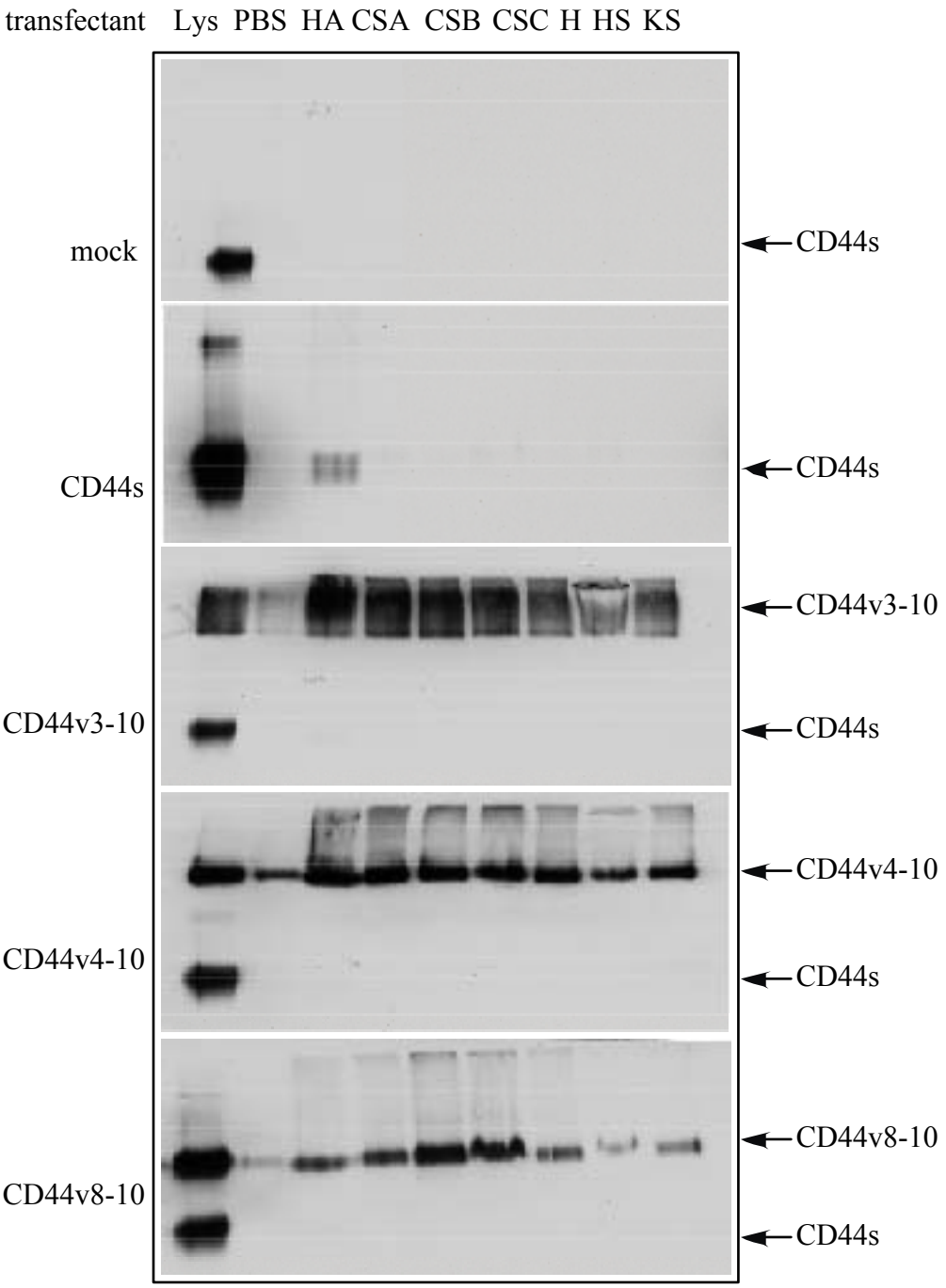


Figure 14. Coprecipitation of CD44 with GAGs and CPC. The mock-, CD44s-, CD44v3-10-, CD44v4-10- and CD44v8-10-transfectants (cultured without Dox, 10^6 cells) were lysed with non-reducing lysis buffer. Cell lysates were mixed with different GAGs (1 μg/μl) or PBS as control, and the GAGs were precipitated by adding CPC to a final concentration of 1%. CPC precipitates of proteins were resolved by SDS-PAGE followed by Western blotting with anti-pan CD44 Ab (IM7). HA: hyaluronate; CSA, B, C: chondroitin sulfate A, B, C; H: heparin; HS: heparan sulfate; KS: keratan sulfate; Lys: cell lysate from 2×10^5 cells.

3.2.4 The regulation of HA binding by CD44 oligomerization

The mere expression of CD44 does not necessarily render cells to bind HA. The HA binding is influenced by additional factors such as variant exon inclusion, clustering and glycosylation.

3.2.4.1 CD44-containing complexes formed in CD44s- and CD44v8-10-transfectants are reduction-sensitive

To examine if CD44 clustering plays a role in LB lymphoma transfectants, we first looked at whether CD44 isoforms could cluster by using a Western blot approach. To resolve CD44 clusters that may be maintained by disulfide bridge formation in SDS-PAGE cellular proteins were disolved in non-reducing lysis buffer (omitting DTT). Endogenous CD44s (transfectants cultured in the prescence of Dox or untransfected cells) migrated in the 75-95 kD (apparent molecular weight (MW)) range (Fig. 15A). Upon ectopic expression of CD44s and CD44v8-10, main bands were found around apparent MW of 75-95 and 130-150 kD respectively, reflecting monomers. Additionally, higher-MW complexes were detected. In CD44s-transfectants they had an apparent MW about 160-180 kD, and in CD44v8-10-tranfectants the apparent MW is about 260-300 kD (Fig. 15A, B).

The high-MW complexes disappeared under reducing conditions since none of our CD44 mAbs worked well in reducing Western blots. We labeled proteins with biotin, immunoprecipitated CD44 with mAb and resolved precipitates on reducing or non-reducing gels. The proteins were then visualized with Avidin-HRPs. This method also showed that the high-MW complex was found in CD44s-transfectants only when the ectopic CD44s was expressed (Fig. 16). In CD44v8-10-transfectant, the band found in previous Western blot was also able to be detected, additionally, another band with a little lower MW was revealed upon removal of Dox. All these three complexes disappeared under the reduction conditions, suggesting that they are linked most likely via disulfide bond. Iodoacetamide was always included in the lysis buffer because it can prevent the cleavage of dimers and inhibit non-specific disulfide interactions.

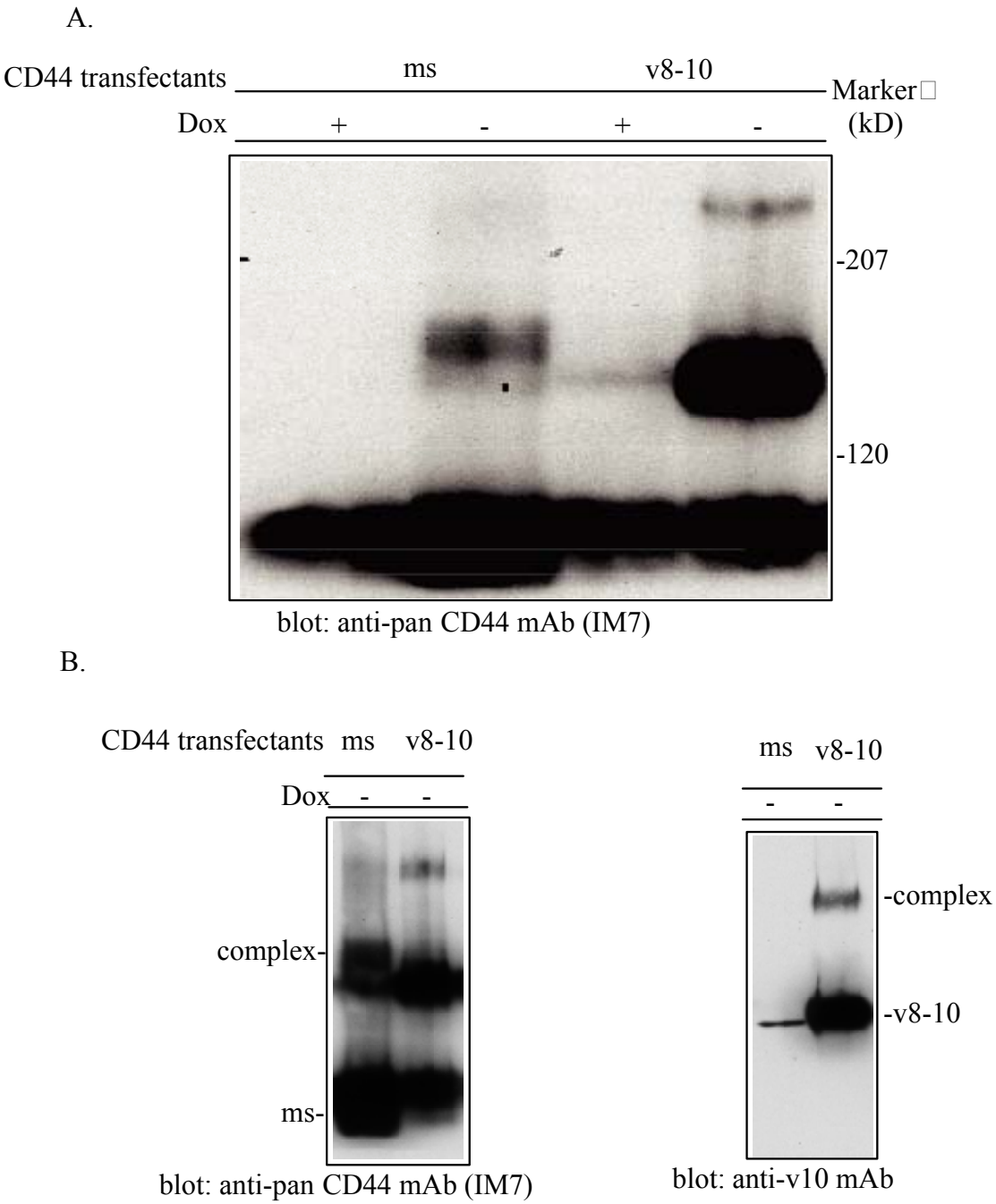


Figure 15. High-molecular-weight (MW) complexes of CD44 can be detected by Western blot. The cells were lysed with non-reducing lysis buffer (125 mM Tris·Cl, pH6.8, 4.1% SDS, 17.4% glycerol, 10 mM iodoacetamide, and 1 mg/100ml bromphenol blue) and the lysates were loaded on SDS-PAGE. After transfered to PVDF membrane, CD44 was probed with the Abs indicated.

In summary, CD44-containing complexes are present in both CD44s- and CD44v8-10-transfectants. These complexes are reduction sensitive and thus may be formed via disulfide linkage.

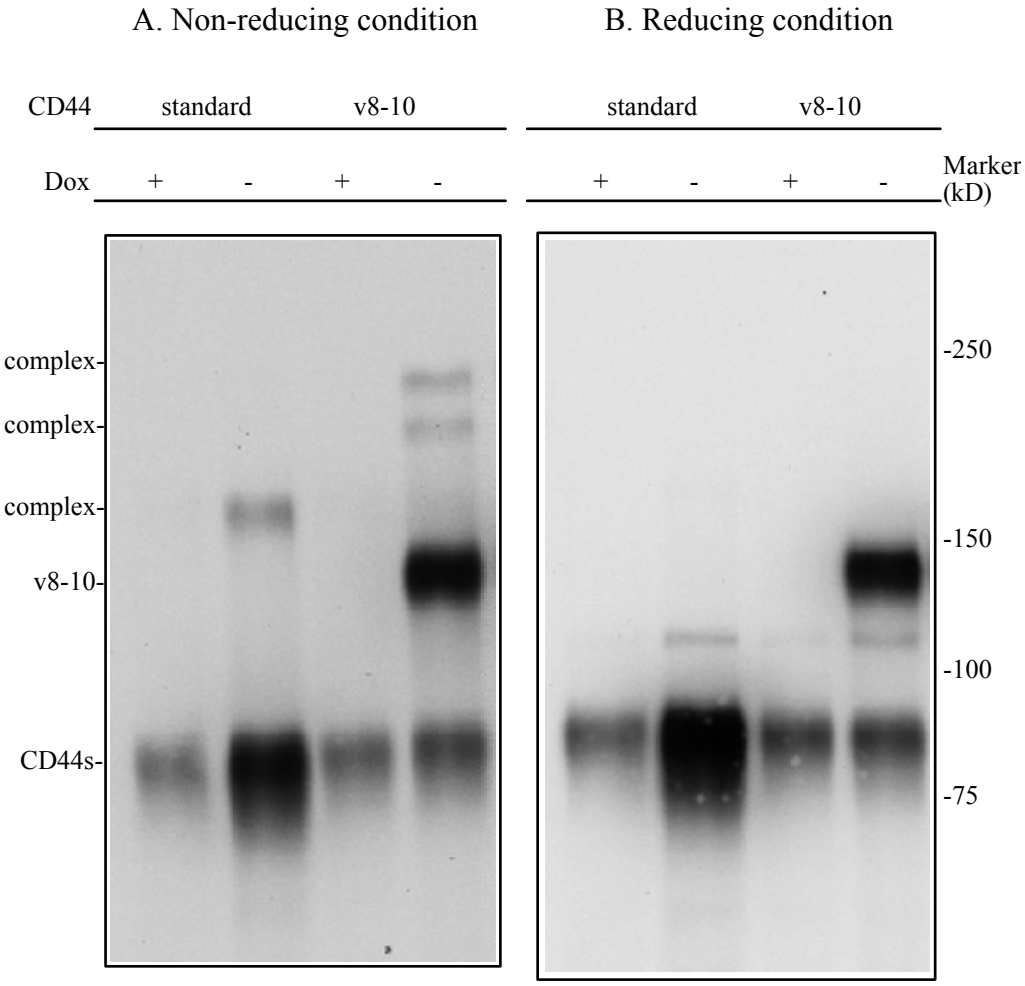


Figure 16. Reduction-sensitive high-order complexes can be detected in CD44s- and CD44v8-10-transfectants. Cell-surface proteins on transfectants were biotinylated and extracted with RIPA buffer. CD44 was precipitated with anti-pan CD44 mAb (IM7), solubilized from the beads with non-reducing (125 mM Tris·Cl, pH6.8, 4.1% SDS, 17.4% glycerol, 10 mM iodoacetamide, and 1 mg/100ml bromphenol blue) (A) or reducing (the same as non-reducing buffer but with 0.15% DTT) (B) lysis buffer and resolved by SDS-PAGE (6%). Blots were probed with NeutrAvidin-HRP.

3.2.4.2 CD44 can homodimerize and heterodimerize

To distinguish whether the high-MW complexes reflect homo or heteromeric CD44 associates or are composed of other proteins besides CD44, we performed a

partial digestion with V8 peptidase *in situ* (Fig. 17). Provided that only CD44 molecules are contained in the complex, the digestion pattern of the complex should be the same as that of CD44 monomers. In case that the complex consists of other proteins together with CD44, one would expect that the digestion of the complex gives rise to peptide fragments differing from that of CD44 monomers. Biotinylated CD44s-containing complexes were excised from the gel, reduced with DTT and digested with V8 peptidase. The digestion pattern was similar to that obtained from CD44 monomers, suggesting that the components in the CD44 high-MW complex are CD44 monomers.

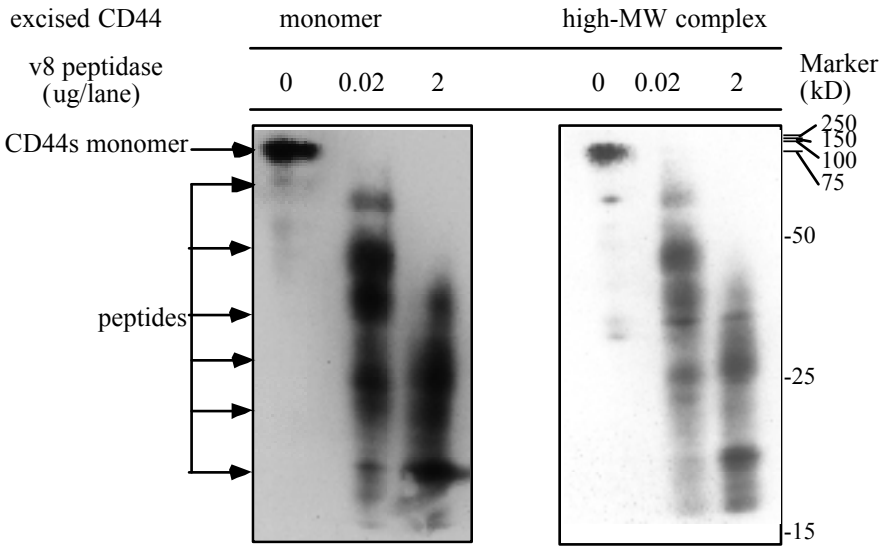


Figure 17. Peptide mapping of CD44 monomers and high-MW complexes. Biotinylated CD44 was immunoprecipitated and resolved in 6% SDS-PAGE. The gel slices containing CD44s monomer and high-order complex were excised from the gel, treated with reducing buffer (125 mM Tris·Cl, pH6.8, 0.1% SDS, 0.l5% DTT) for 1 hr and loaded together with V8 peptidase (concentrations are indicated) onto a gel with a 5-cm stacking gel and 15% separating gel. When the proteins had entered the stacking gel and the dye marking the front was at the bottom of the stacking gel, the current was turned off and proteins were digested *in situ* for 0.5 hr. After resolution of the peptides and transfer, blots were probed with NeutrAvidin-HRP.

Another approach to identify the components of complex is the nonreducing /reducing two-dimensional gel analysis of the complex. The biotinylated proteins precipitated with CD44 antibodies were run under nonreducing conditions in the first dimension and then under reducing conditions in the second dimension. In the first

dimension, high-MW complex and CD44 monomer are seperated according to their molecular sizes. When we dissociate the complex with reducing lysis buffer and run on the gel in the second dimension, we expect to see a spot that moves at the same position as CD44 monomer in case that the complex is a CD44 dimer, or several spots that run at different positions in case the complex is composed of other proteins besides CD44. Fig. 18A shows the bands representing CD44s-containing complex and CD44 monomer under nonreducing conditions in both dimensions line up in a diagonal. After dissociation of high-MW complex under reducing conditions in the second dimension, we observed that besides CD44s monomer, one spot (shown in the left) migrated at the same position as monomer, suggesting that the complex is CD44s homodimer (Fig. 18B).

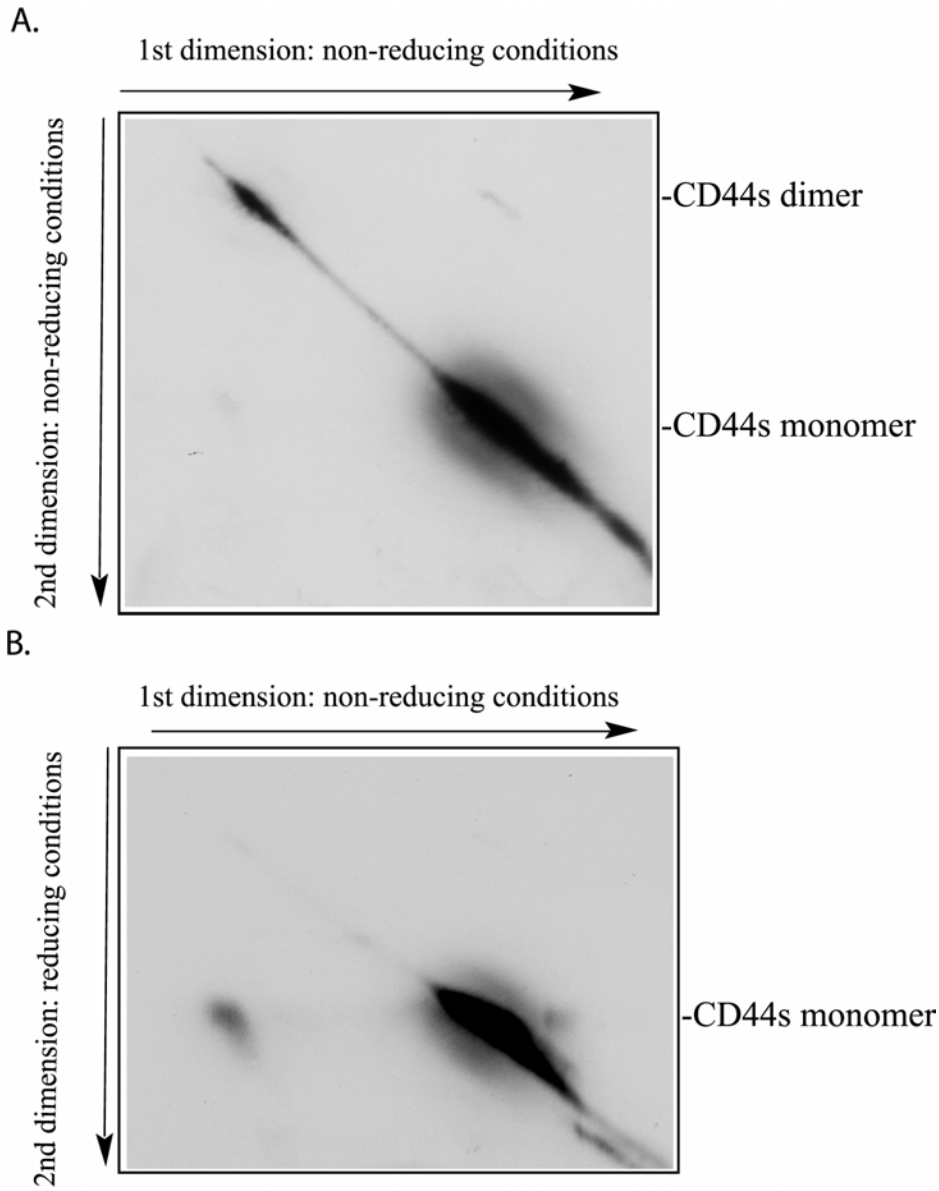


Figure 18. Homodimerization of CD44s. Immunoprecipitates from biotinylated CD44s-transfectants were resolved in a 6% non-reducing tube gel in the first dimension. After incubation of the tube gel with non-reducing (A) (125 mM Tris·Cl, pH 6.8, 0.1% SDS) or reducing buffer (B) (the same as non-reducing buffer but with 0.5% DTT), the proteins within the tube gel were run into a 6% SDS-PAGE gel in the second dimension. After blotting to PVDF membrane, proteins were probed with NeutrAvidin-HRP.

Instead of two-dimensional gel analysis, we also performed the experiment in two-step way. The CD44 complexes and monomers were seperated by the first gel. The gel slices corresponding to the complexes were then excised from the gel and

subsequently reduced. Finally, the gel slices were resolved in the second gel. The complexes formed in CD44s- and CD44v8-10-transfectants were shown in Fig. 19A. Upon reduction, the high-MW complex of CD44s-transfectants migrated as a single band at the same position as CD44s monomer control (Fig. 19B). The high-MW complex with higher MW formed in CD44v8-10-transfectants contained only CD44v8-10, whereas the high-MW complex with the somewhat lower MW contained CD44v8-10 together with CD44s, suggesting CD44v8-10 can both form homo and heterodimers.

A. Non-reducing condition B. Reducing condition

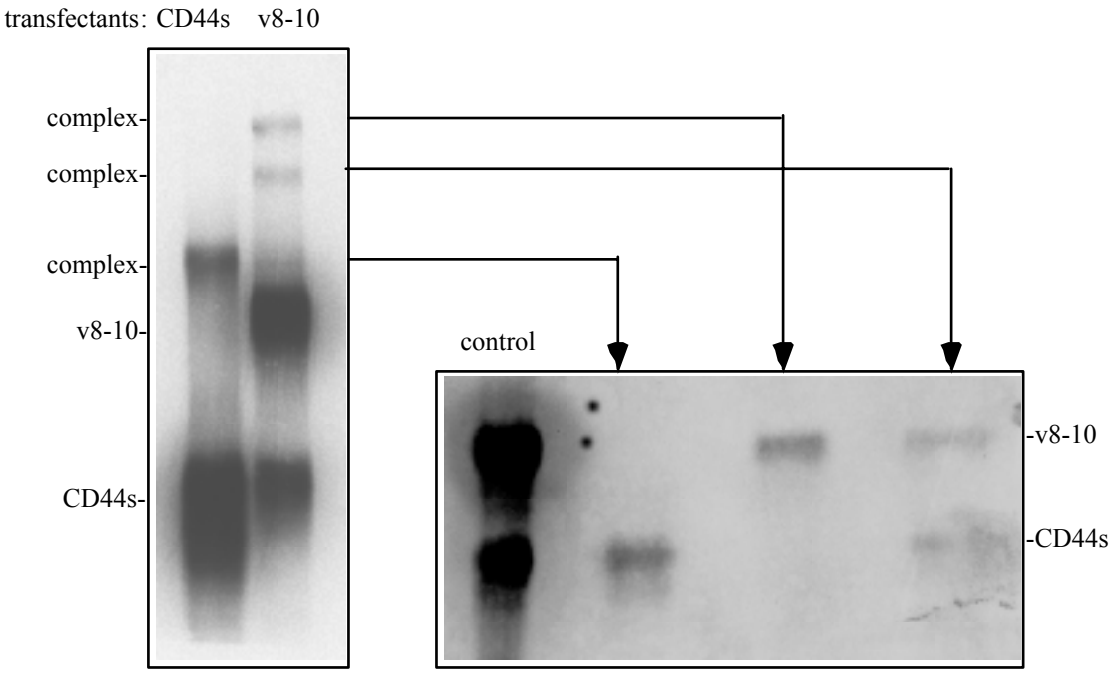


Figure 19 Homodimerization of CD44s and homo- and heterodimerization of CD44v8-10. A. CD44s and CD44 v8-10 transfectants were biotinylated, and CD44 were immunoprecipitated with anti-pan CD44 mAb (IM7) and loaded on 6% SDS-PAGE. After transfer, Blots were probed with NeutrAvidin-HRP. **B.** The gel slices corresponding to the bands shown in Fig. A were excised from the gel and treated with reducing buffer (0.5% DTT, 125 mM Tris·Cl, pH 6.8, 0.1% SDS) and reloaded again on a second SDS-PAGE gel. After transfer, blots were probed with NeutrAvidin-HRP.

So far the experiments are based on cell surface labeling with sulfosuccinimidobiotin which is a water-soluble and plasma membrane impermeable reagent. It can only label the extracellular domains of cell surface molecules but not

their transmembrane and cytoplasmic domains and cytosolic proteins. Even some cell surface molecules appear to be not labeled with biotin (Bourguignon et al., 1997). To avoid these drawbacks of biotinylation procedure, we metabolically labeled transfectants with ^{35}S methionine. After immunoprecipitation with CD44-specific mAbs, CD44s-containing complexes and monomeres were resolved under non-reducing conditions and seperated by SDS-PAGE. The gel slices were excised from the gel and again subject to V8 peptide maping. The digestion patterns of CD44s complexes and monomers are the same, thus proving that there is no other prtoteins besides CD44s in the complex (Fig. 21 and data not shown).

In summary, high-MW complexes formed in CD44s-transfectants are CD44 homodimers and those in CD44v8-10-transfectants are homo and heterodimers. All the dimers are reduction sensitive and most probably formed through disulfide bond interaction.

3.2.4.3 Higher-MW oligomers are detectable via chemical cross-linking

In addition to dimerization, CD44 can cluster to oligomers. This type of interaction among CD44 molecules has been proposed to be required for binding of cells to HA (Lesley et al., 1993; Sleeman et al., 1996). To investigate whether CD44 clustering exists also in LB cells, we used chemical crosslinkers which might stabilize oligomeric complexes formed on cells. Among a number of crosslinking reagents we found two membrane-impermeable cross-linkers, DTSSP and BS^3, which worked efficiently in LB cells in that they crosslinked the ectodomains of CD44 molecules. In cells that express CD44s ectopically, upon treatment with DTSSP and BS^3, we observed bands of higher MW in addition to CD44s dimers (Fig. 20), while such complexes were not detected in untransfected cells treated with the crosslinking agents. In PBS-treated cells, higher-MW complexes were not seen as well.

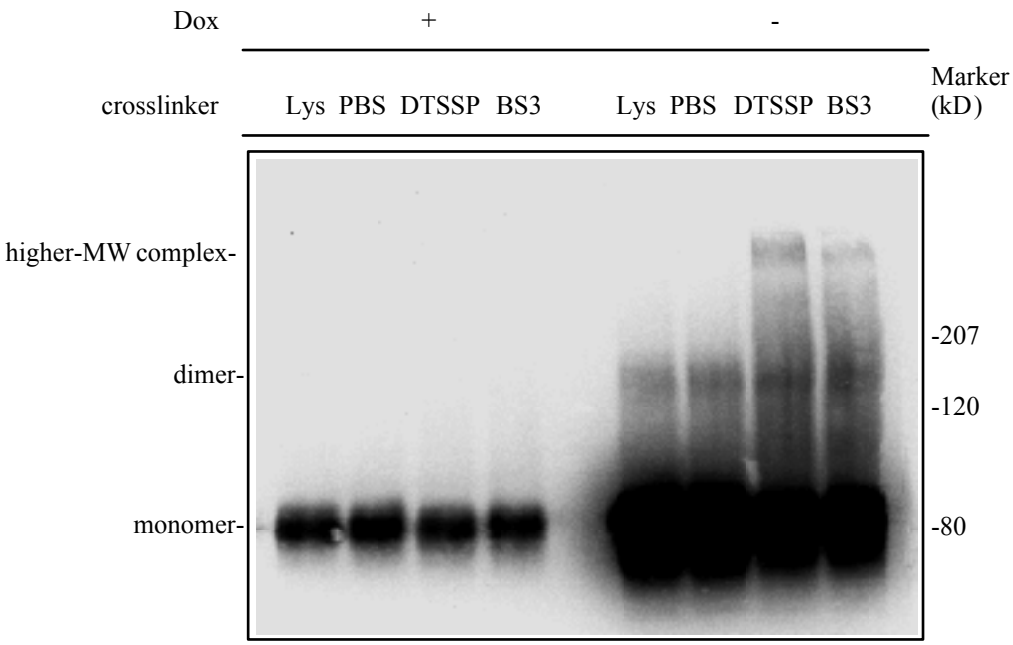


Figure 20. CD44s higher-MW complex can be revealed by chemical crosslinking. CD44s transfectant (cultured with or without Dox) were incubated with 2 mM crosslinkers (in PBS) or PBS control for 1 hr on ice. Unreacted crossliners were quenched by incubation with 150 mM Tris·Cl, pH 7.4 for 15 min. Then the cells were harvested, washed twice with PBS and lysed with non-reducing lysis buffer. After transfer, CD44 was detected with anti-pan CD44 Ab (IM7).

The homo and heterodimers of CD44 isoforms formed in LB cells are reduction-sensitive, suggesting they might be formed through covalent disulfide linkage. CD44 has six Cys residues in the N-terminal region which is conserved among species and may form a single globular domain, one Cys (Cys286) in the transmembrane domain and one Cys (Cys295) in the cytoplasmic domain (Lesley and Hyman, 1998; Naor et al., 1997).

3.2..4.4 V8 peptide mapping of CD44 monomer and dimer

To find out the Cys residue that could be responsible for the disulfide interaction between CD44 molecules, we performed V8 peptide mapping of CD44s monomer and dimer. The rationale of this approach is that if CD44 forms dimer via intermolecular disulfide bonds, the peptidase digestion of CD44 dimer and monomer under non-reducing conditions would give rise to digestion patterns with some peptides migrating differently. Therefore V8 peptide mapping would provide a gross

hint whether CD44 molecules dimerize via covalent association or not. The cells were metabolically labelled with ^{35}S methionine and CD44 was immunoprecipitated with CD44-specific antibody. The proteins were then seperated on a first gel from which the gel slices containing CD44 dimers and monomers respectively were excised. Those slices were then loaded on a second gel together with V8 peptidase, digested and the peptides were resolved. The digestion pattern of CD44s dimer is very similar to that of CD44s monomer (Fig. 21) although there might be slight difference. One band (marked by square) present in the peptide map of dimer seems missing in the peptide map of monomer, suggesting that possible covalent interaction exists among CD44 molecules.

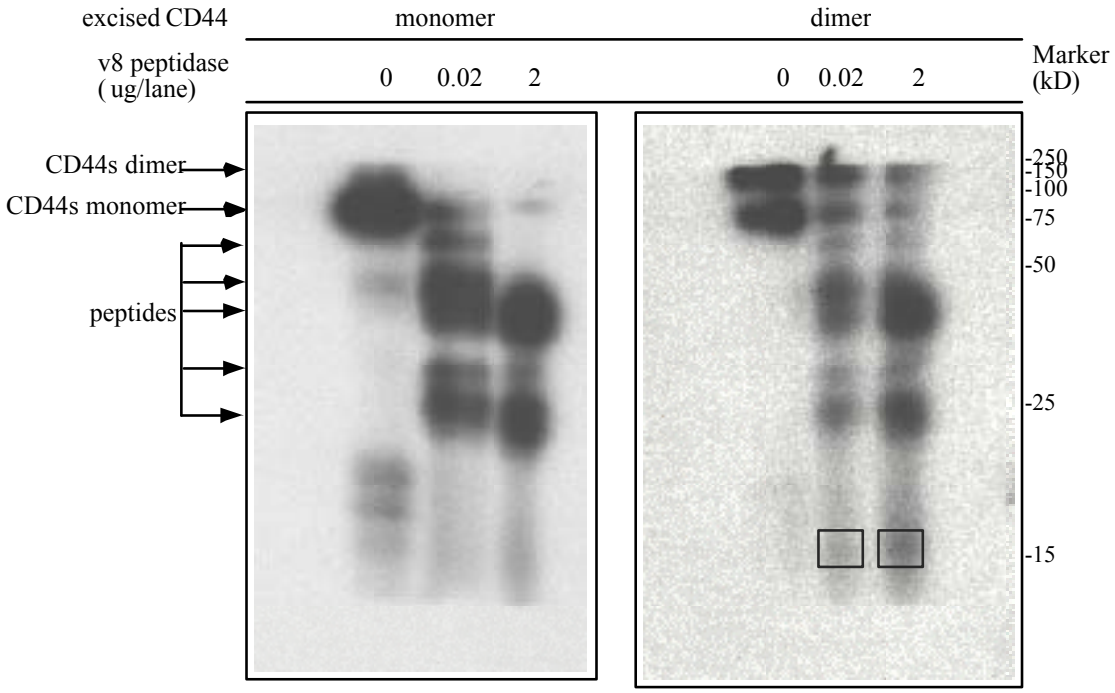


Figure 21. Peptide mapping of CD44s monomer and dimer. Metabolically labeled CD44 was immunoprecipitated and resolved in 6% SDS-PAGE. The gel slices containing CD44s monomer and dimer were excised from the gel, treated with non-reducing buffer (125 mM Tris·Cl, pH6.8, 0.1% SDS) for 0.5 hr to equilibrate the gel slice and loaded together with V8 peptidase (the amounts are indicated) onto the second gel with a 5-cm stacking gel and 15% separating gel. When the dye migrated close to the bottom of the stacking gel, the current was turned off and the proteins were digested by V8 peptidase *in situ* for 0.5 hr. After fractionation, the gel was treated with Amplify (Amersham) to intensify the radioactivity signal, dried under vacum and exposed with an X-ray film.

3.2.4.5 Cys286 in transmembrane domain is not the target for mouse CD44 dimerization

In human CD44, Cys286 in the transmembrane domain is believed to be the target of CD44 dimerization (Liu and Sy, 1997). The Cys at a similar position exists also in the murine CD44 proteins, therefore we examined an involvement of this Cys by mutation analysis. We mutated Cys286 into Ala in CD44v8-10 (Fig. 22) and transfected this mutant into the founder cell line. Several clones were analysed for CD44 dimerization by Western blot (Fig. 23) and HA binding by FACS analysis (Fig. 24). Suprisingly, CD44v8-10 (C286A) mutant formed dimer to the same extent as observed for wild type CD44v8-10 in all of the four representative clones, HA binding capacity of mutant-expressing cells was also not compromised. CD44s (C286A)-expressing clones also showed unimpaired dimer formation and HA binding (data not shown).

mCD44s/v8-10WT:

LIILASLLALALILAV**C**IAVNSRRRCGQKKKLVINSGNG

transmembrane domain part of cytoplasmic tail

mCD44s/v8-10(C286A)mutant:

LIILASLLALALILAV**A**IAVNSRRRCGQKKKLVINSGNG

transmembrane domain part of cytoplasmic tail

Figure 22. Illustration of Cysteine to Alanine mutation.

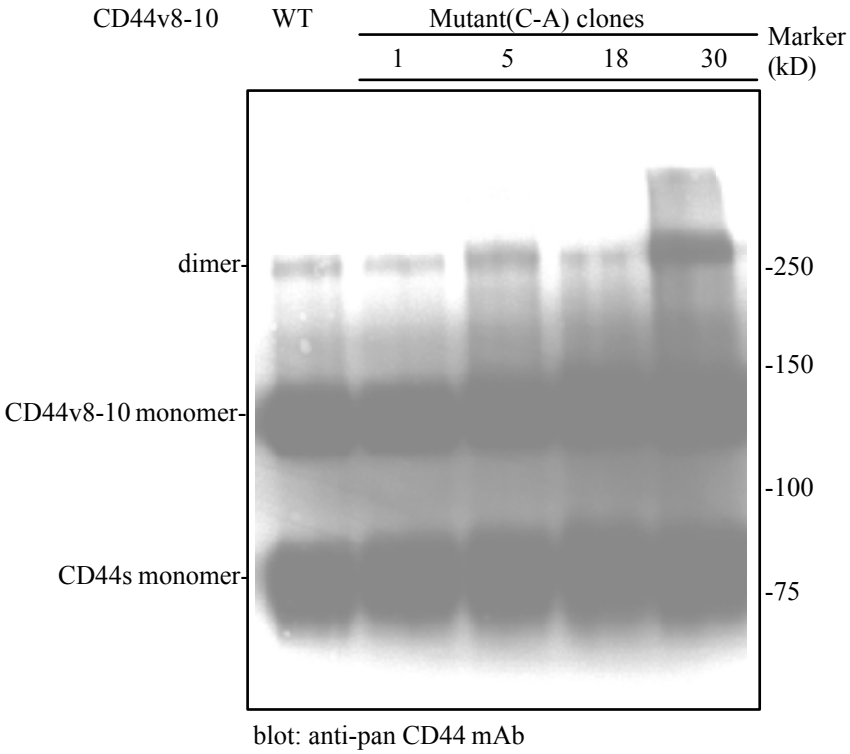


Figure 23. Dimer formation is not changed by Cysteine286 mutation. Cell lysates from CD44v8-10 and mutant (C286A) transfectants were loaded and run on SDS-PAGE. After transfer to PVDF membrane, CD44 was probed with anti-pan CD44 mAb.

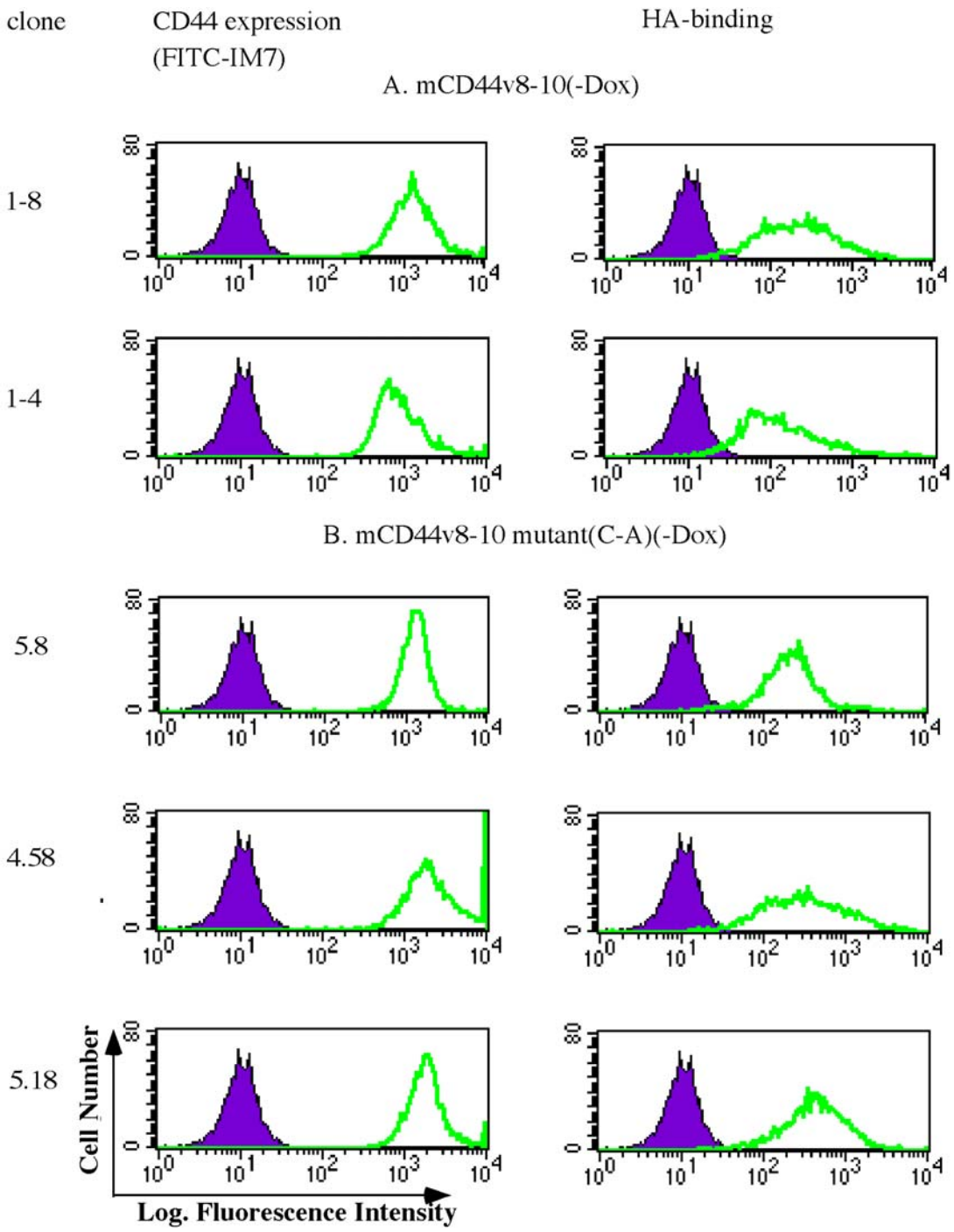


Figure 24. HA-binding is not altered by Cys mutation in CD44v8-10 transfectants. Individual clones from wild type (A) and mutant CD44v8-10 (B) transfectants (cultured without Dox) were used for the analysis of CD44 expression (anti-CD44v10 staining, shown in the left panels) and HA-binding (FITC-HA staining, shown in the right panels) by FACS analysis.

3.2.4.6 Functional consequence of CD44 dimerization and oligomerization

To directly study the functional significance of CD44 dimerization or oligomerization in LB cells, we made use of a conditional dimerization system.

a) Conditional dimerization and oligomerization system

In the conditional dimerization system, the dimerization domain FKBP is fused to CD44 cytoplasmic tail and the fusion protein is overexpressed in cells. CD44 Dimer- or oligomerization is induced upon treatment of transfected cells with dimerizer (AP20187). CD44v8-10-FKBP and -3FKBP were constructed (as demonstrated in Fig. 3) and introduced into the founder cell line.

b) Characterization of LB cells expressing CD44v8-10 fusion protein with dimerization domains

A Western blot analysis of clones from CD44v8-10WT and CD44v8-10-(3)FKBP revealed that CD44v8-10(3)FKBP proteins with a slightly higher molecular weight than that of CD44v8-10WT, as expected from the addition of the FKBP sequences (Fig. 25A). The chimeric CD44v8-10 proteins can also be detected by anti-hemagglutinin (Fig. 25B) and anti-FKBP (Fig. 25C) antibodies.

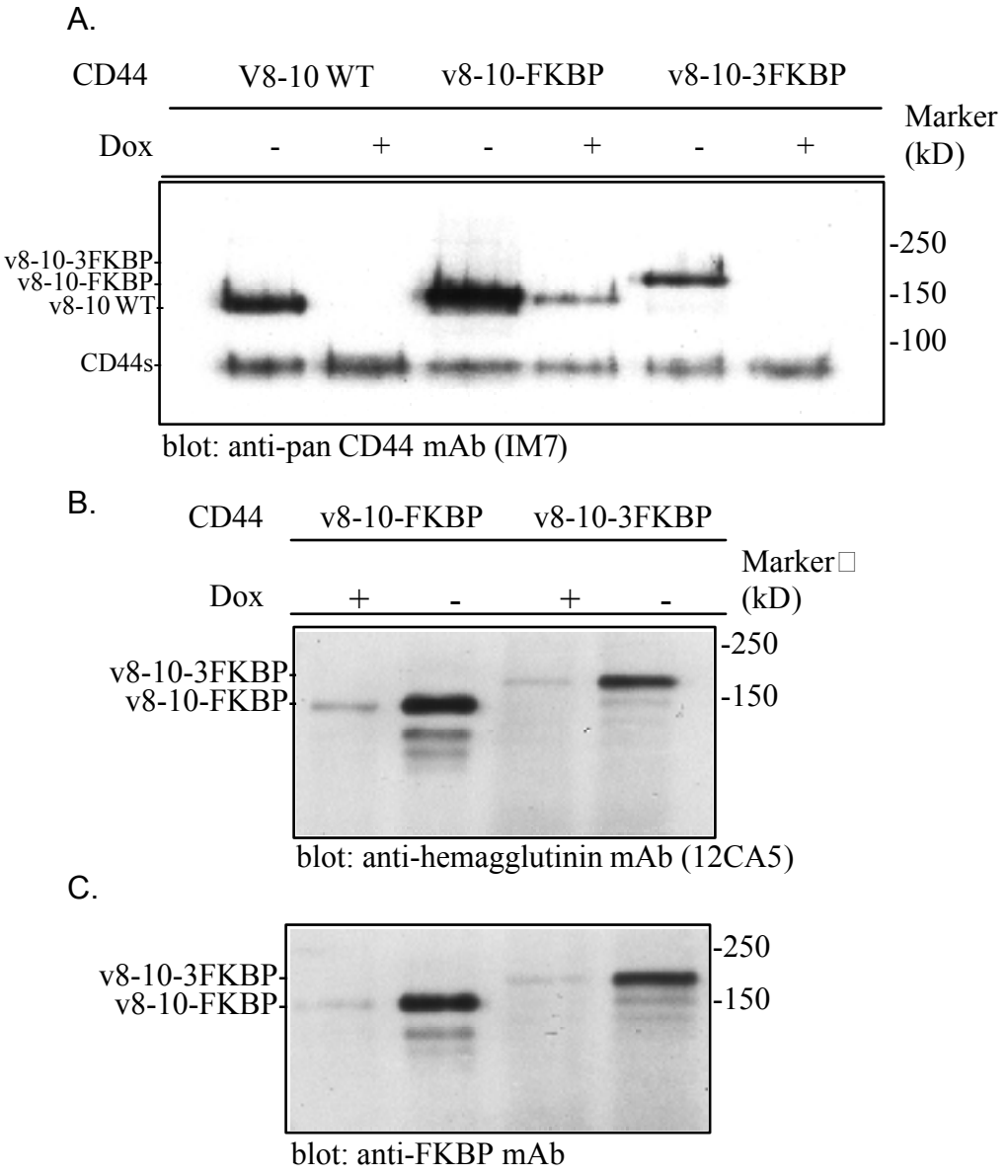


Figure 25. Western blot detection of CD44 in lysates of CD44v8-10 wild type (WT), CD44v8-10-FKBP and CD44v8-10-3FKBP transfectants. A. Cells were directly lysed under non-reducing conditions and the proteins were fractionated by SDS-PAGE. After transfer to PVDF membrane, CD44 were detected by IM7. B and C. CD44 proteins were immunoprecipitated from transfectants with IM7 followed by solubilization under reducing conditions. The proteins were then Western blotted and probed with anti-hemagglutinin Ab (12CA5) (B) or anti-FKBP mAb (C).

c) Induced clustering of CD44 has moderate effect on HA binding

We examined by Western blot analysis whether dimer inducer (AP20187) could indeed induce the oligomerization of chimeric CD44. To stablize the oligomers

dimerizer-induced cells were further treated with crosslinker (DTSSP). Upon treatment with both dimerizer and crosslinker, we observed much stronger bands of high MW in cells that express CD44v8-10-FKBP and v8-10-3FKBP than in cells only treated with crosslinker (Fig. 26). In cells expressing CD44v8-10, the amounts of high-MW complexes are similar whether or not the cells were treated with AP20187.

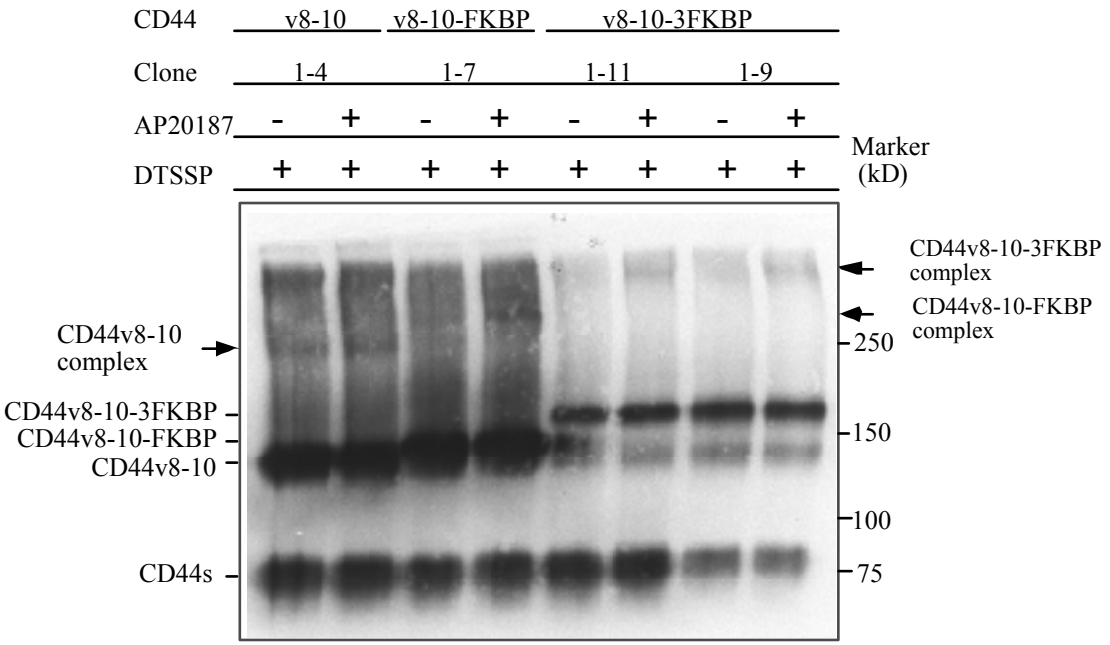


Figure 26. Crosslinking of CD44 on cells treated with or without dimer inducer (AP20187). 5×10^6 cells (cultured in the absence of Dox) were harvested, washed with PBS twice, and treated with 1 μM dimerizer (AP20187) or vehicle buffer at room temperature. After 20 min, cells were incubated with 2 mM crosslinker (DTSSP) for 1 hr on ice. Unreacted crosslinkers were quenched by incubation with 150 mM Tris·Cl, pH 7.4 for 15 min on ice. Then the cells were harvested and lysed with 200 μl non-reducing lysis buffer. 50 μl of the cell lysate was loaded on 6% SDS-PAGE gel. After transfer to PVDF membrane, CD44 was detected with anti-panCD44 mAb (IM7).

The induction of oligomerization by treating the cells with AP20187 increased HA binding of these cells in a dose dependent fashion (Fig. 27). In CD44v8-10

transfectants AP20187 had no influence on HA binding.

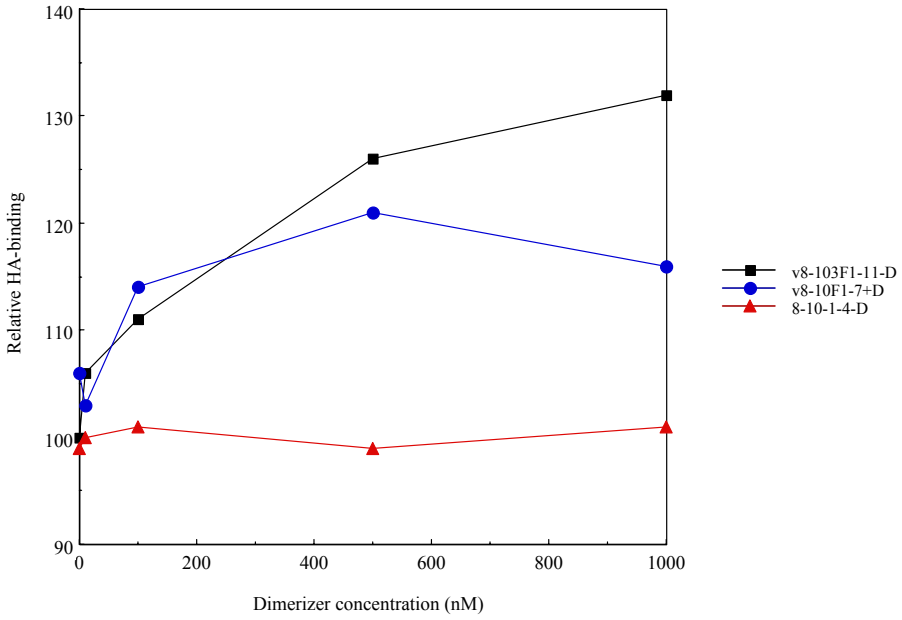


Figure 27. Dose-dependent effect of dimerizer AP20187 on HA binding. CD44v8-10WT, CD44v8-10-3FKBP (clone1-4 and 1-11 respectively, cultured without Dox) and v8-10-FKBP (clone 1-7, cultured with Dox) transfectants were treated with AP20187 of different concentrations or with ethanol control for half an hour at 37ºC, and harvested. The HA binding was analysed by FACS and expressed as the relative HA binding (100%): HA binding of cells treated with AP20187 / HA binding of cells treated with ethanol control.

Examinatioin of various clones transfected with either CD44v8-10-FKBP or CD44v8-10-3FKBP revealed that increased HA binding upon AP20187 treatment (Table 1 and 2). The enhanced HA binding was easily detected in CD44v8-10-3FKBP transfectants. Upon treatment with AP20187, CD44v8-10-3FKBP transfectants showed modestly higher HA binding than vehicle control-treated cells, while the control cell lines (CD44v8-10) did not have such an effect. The effect of binding seems to correlate with the amount of CD44 expressed in the various clones.

Table 1 The effect of dimer inducer on HA binding in mCD44v8-10-3FKBP transfectants

clone name	control	5.4	1.1	1.11	6.3	6.5	1.9
CD44 amount	33	36	56	63	99	110	124
HA binding*(%)	97±2	115±2	132±4	122±3	115±4	116±3	117±6

Table 2 The effect of dimer inducer on HA binding in mCD44v8-10-FKBP transfectants

clone name	control	3.1	3.2	1.10	2.3	1.12	1.7
CD44 amount	5	55	65	94	186	247	362
HA binding*(%)	104±1	118±3	107±2	97±6	122±3	106±5	100±8

The CD44 amount shown here is the Mean Fluorescence Intensity (MFI) .The HA binding of cells treated with vehicle control was set arbitrarily as 100%. The HA binding* is the relative HA binding of the cells treated with dimer inducer compared to that of the cells treated with vehicle control.

3.3 The role of CD44 variant isoforms in LB lymphoma development

CD44 variant isoforms play roles in tumorigenesis and metastasis. For example, CD44 variants containing CD44 v6 have been shown to confer metastatic behavior on rat pancreatic carcinoma cells (Sleeman et al., 1996). CD44 v5 and v6 expressions have been proposed to be an early tumor marker and a prognostic factor of human colorectal carcinoma respectively (Naor et al., 1997). Increased CD44 v7/8 expression is correlated to the progression of human cervical carcinoma (Naor et al., 1997). To study the roles of CD44 variants in LB lymphoma development, we injected LB cells conditionally expressing different CD44 variants into the mice, controled CD44 expression by providing the mice with or without Dox and monitored both the local tumor growth and metastasis into lymph node and spleen.

3.3.1 The effect of CD44 variant isoforms on lymphoma growth

Because LB lymphoma cells arouse from a BALB/c strain mouse (Zahalka et al., 1995)**,** we firstly injected LB cells into syngeneic mice. Tumor incidence was found to be much lower in LB transfectant-injected syngeneic BALB/c strain mice compared to that of LB parental cell-injected mice. 80-100% of the mice injected with parental cells developed tumors after about 2 weeks, whereas in transfectant-injected group, the tumor incidence was only 20-40%, rendering us unable to have enough samples for statistical analysis.

LB transfectants express high-level tetracycline-controled transactivator (tTA) which consists of Tet repressor from *E. Coli* and VP16 protein of Herpes Simplex Virus (Gossen and Bujard, 1992; Gossen et al., 1995). High expression of these proteins could cause immunological rejection from BALB/c strain mice and thus low tumor take rate, therefore we decided to use immunodeficient MF1 strain nude mice for the injection of LB transfectants alternatively. In the first attempt to study the roles of CD44 variants, we injected CD44v4-10 transfectants into nude mice and mock transfectants as a control. LB transfectants were found to be capable of consistently generating tumors in 80-100% nude mice injected. Fig. 28 is one representative result showing that tumors grew rapidly in both the group of mice injected with mock and

those with CD44v4-10 transfectants. In CD44v4-10 transfectant-injected group, the tumor size of mice provided with Dox is similar to that of the mice without Dox. This is also the case in the mice injected with mock transfectants. To test that the conditional expression system worked properly *in vivo,* we checked CD44 expression by FACS analysis of the LB cells isolated from injected mice at different time points. We found that, on the one hand, ectopic CD44 expression can be turned on spontaneously *in vivo* after 3 days post injection and reaches high level after 5 days, on the other hand, ectopic CD44 expression can be kept off continously by Dox *in vivo* (data not shown), implicating Tet-off system *per se* works *in vivo*.

3.3.2 The effect of CD44 variant isoforms on lymphoma metastasis

No difference was found concerning the metastatic ability of LB cells with or without CD44v4-10 expression regulated by Dox. Both the CD44v4-10 transfectants that expressed high-level CD44v4-10 in the absence of Dox and those that expressed low-level in the prescence of Dox were able to invade into lymph node (but not spleen) when the tumors were big at the final stage of tumor development, while at early stage when tumor size was small, the invasion was undetectable in both groups. Their incidences of invasion into lymph node were similar (80-100%).

No enhanced tumor growth and metastasis due to the overexpression of CD44v3-10 or v8-10 was observed by injecting these two LB transfectants (data not shown).

In summary, CD44v3-10, v4-10 and v8-10 isoforms were found neither to be required at the onset stage of LB lymphoma tumorigenesis nor to be able to confer growth and metastasis advantages on LB lymphoma cells *in vivo*.

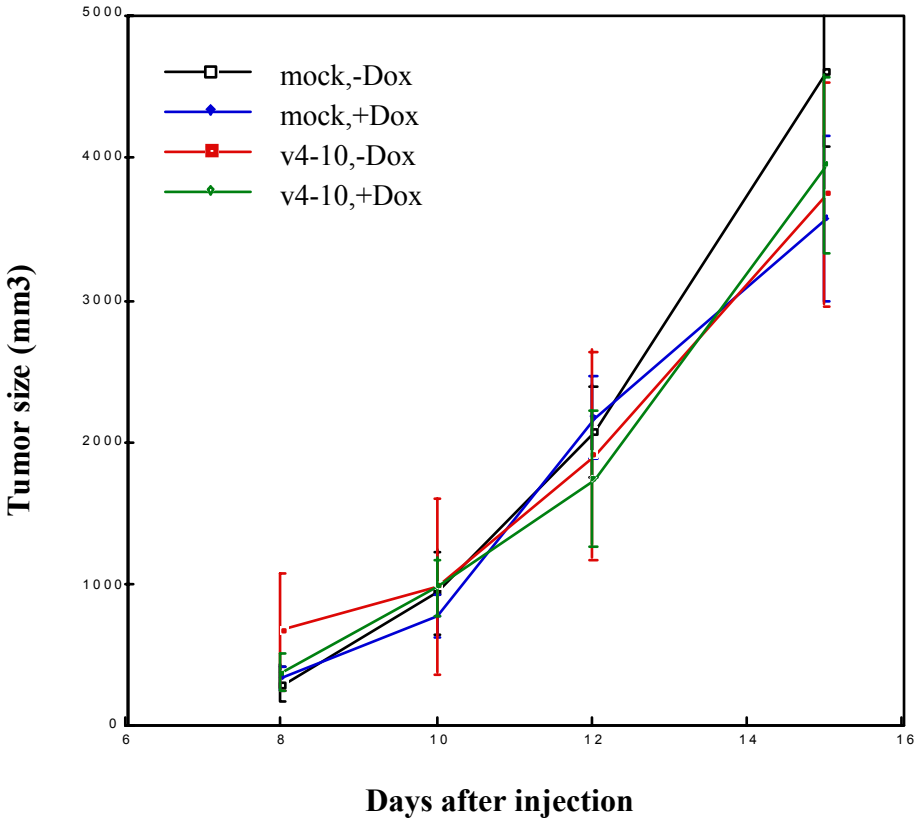


Figure 28. *In vivo* LB lymphoma growth. 3×10^6 cells (in 0.1 ml PBS containing 1 µg/ml Dox) were injected subcutaneously into the left flank of MF1 nude mice. After injection, the mice were seperated into two groups, one fed with normal drinking water and the other with Dox (100 µg/ml) in drinking water. The tumor volumes were measured and calculated according to the formula: volume=length×width^2/2. The mean values of tumor volume were ploted against Days after injection.

PART FOUR

DISCUSSION

In this study, I conditionally regulated CD44v3-10, v4-10, v8-10 and CD44s expression in the LB lymphoma cell line using the Tet-off gene expression system. Overexpression of these four CD44 isoforms increased HA binding of LB cells no matter which isoform was used. CD44 isoforms bound differentially to other GAG ligands. CD44v3-10, v4-10 and v8-10 showed high binding affinity to chondroitin A, B and C, and low affinity to heparin, heparan sulfate and keratan sulfate, whereas CD44s could not bind to these GAGs. Among these three variant isoforms, the binding ability of CD44v3-10 was the highest. HA binding of CD44 was regulated by CD44 clustering. Both CD44s and CD44v8-10 formed reduction-sensitive complexes in LB cells. The complexes were CD44 homooligomers or heterooligomers composed of different isoforms. Cys286 in the CD44 transmembrane domain was not responsible for the formation of reduction-sensitive dimer or for the enhanced HA binding in LB cell line. A conditional dimerization system provided direct evidence that induction of CD44 clustering increased the HA binding of CD44. Finally, I studied the role of CD44 in LB lymphoma development, however, I found that CD44v3-10, CD44v4-10 and CD44v8-10 isoforms did not influence the outgrowth of LB lymphoma nor their metastatic spreading.

4.1 Regulation of HA and other GAG binding by variant exon inclusion

It has been reported that CD44s bound to HA differently from CD44 variant isoforms. For example, in a pancreatic carcinoma cell line, cells expressing CD44 variants bound HA more strongly than CD44s expressor (Sleeman et al., 1996). On the contrary, upon overexpression in a B cell lymphoma cell line (Namalwa), CD44s conferred efficient HA binding while CD44 variants failed to do so (Bartolazzi et al., 1995; Jackson et al., 1995). However, it has also been shown that both CD44s and CD44 variant isoforms have similar ability to bind HA in a T cell lymphoma cell line (He et al., 1992). Consistent with this finding, my results demonstrate that in the LB

cell line expression of CD44 variants (CD44v3-10, v4-10 and v8-10) as well as of CD44s conferred comparable HA binding. An explanation for the inconsistent reports about the effect of overexpression of CD44s and CD44 variant isoforms on HA binding might be that there exists a cell type specific effect regarding the HA binding of CD44. This cell type specific difference might be attributable to difference in posttranslational modification of CD44 molecule.

Regarding binding to other glycosaminoglycans (GAGs) than HA, CD44 variants were stronger binders than CD44s, consistent with previous reports (Moll et al., 1998; Sleeman et al., 1997). The combination of CD44 v6 and v7 exons was the minimal variant sequence required for the binding of CD44 variants to chondrointin sulfate (CS) A, B, and C, heparin (H) and heparan sulfate (HS) upon overexpression in a rat pancreatic carcinoma cell line (Sleeman et al., 1997). CD44v4-10 expressed on a hemopoietc progenitor cell line bound strongly to CSA (Moll et al., 1998). CD44s did not bind to GAGs in these two reports. I compared GAG binding ability of different CD44 isoforms expressed in LB lymphoma cells and found that CD44v4-10 bound to CSA, B and C, H, HS and keratan sulfate (KS). Nevertheless, CD44v4-10 is not the strongest binder. The inclusion of exon v3 (CD44v3-10) dramatically enhanced the GAG binding capacity of CD44 molecule. It is not clear yet that how inclusion of CD44 v3 exon can influence GAG binding, however, since the link module is essential for binding of CD44 to GAGs (Kawashima et al., 2000; Sleeman et al., 1997), it is plausible that the incorporation of exon v3 affects the structure of the link module. In the context that many ligands in the ECM or at the cell surface are modified by CS, H or HS (Ruoslahti, 1989), it is tempting to speculate that CD44 may perform its multiple functions by interacting with the GAG side chains of such proteins. Indeed, recently, it was found that CD44 can bind to the GAG-modified proteins aggrecan and versican through interaction with the GAG side chains (Fujimoto et al., 2001; Kawashima et al., 2000).

One conflicting point is that CD44s bound HA less good in CPC experiment as compared with CD44 variants, whereas in FACS analysis, binding was similar for all isoforms. One explanation for this contradiction could be that in CPC precipitation, binding is measured in cell lysate, while FACS analysis involves intact cells. The

organization of CD44 molecules in the plasma membrane might have an impact on HA binding. I also found that the amount of CD44 on cell surface played a predominant role in mediating ligand engagement in the LB lymphoma cell line. High CD44 density in the membrane may promote the formation of CD44 aggrecates, hence enhances HA binding. Reports from other groups agree with this point, for example, CD44s containing truncated cytoplasmic tail had a rather low HA binding ability, but when its density was high enough, it changed to a HA binder (Perschl et al., 1995). CD44s-Ig fusion proteins, upon immobilization on beads, resemble binding of intact cells to HA. Immobilized CD44-Igs achieved HA binding when it reached certain amount regardless that they were secreted from HA-binding inactive or inducible cell lines (English et al., 1998). Changes in density of CD44s in the cell membrane could also explain the change of cells from weak to strong HA binders upon treatment with PMA (Liu and Sy, 1997; Liu and Kaczmarek, 1998; Sionov and Naor, 1998). The upregulation of CD44 induced by PMA could be one of the regulatory mechanisms by which PMA-treated cells achieved higher HA binding ability.

4.2 CD44 clustering

In this study, complexes comprising CD44 were identified in LB cells. Are these complexes just containing CD44 molecules or other molecules in addition to CD44 since, for example, one member of the epithelial growth factor receptor (EGFR) family erbB2 was shown to associate with CD44 on cell surface (Bourguignon et al., 1997)? My data strongly argue that these CD44-containing complexes are CD44 oligomers (Fig.?). Oligomer formation is not the exclusive property of CD44s or CD44 variant isoforms. In LB lymphoma cells, I found that both CD44s and CD44 variant isoform formed oligomers. Data from other studies support this point. For example, CD44 variants formed CD44 oligomer in a rat pancreatic carcinoma cell line (Sleeman et al., 1996), while in human Jurkat transfectants CD44s was demonstrated to be able to dimerize after the PMA stimulation (Liu and Sy, 1997).

Although we can demonstrate that CD44 proteins cluster on cells, the mechnism responsible for CD44 clustering is ill understood. A matter of controversion is the

question which domain and/or amino acid residues of CD44 molecules are critical for aggregation. One Cysteine (Cys) inside the transmembrane domain (C286) was considered to be crucial for human CD44s dimer formation which was linked by disulfide bond and induced by PMA stimulation. Mutation of this Cys to alanine (C286A) abolished dimer formation (Liu and Sy, 1997). Although the transmembrane domain of CD44 and particularly this Cys is conserved between human and mouse, this Cys seemed to be irrelevant for CD44 aggregation in a murine T cell line (Li et al., 1998). My result also argued against the requirement of C286 for clustering. After transfection of mutants in this Cys (C286A) of CD44s (data not shown) or CD44v8-10, LB cells displayed unimpaired dimer pattern. Regarding the question which domain is necessary for dimer formation, it has been reported that the cytoplasmic domain was not involved in clustering of CD44 variants in rat pancreatic cells and of CD44s in several cell lines (Li et al., 1998; Sleeman et al., 1996). There are 6 Cys residues in the extracellular domain and 1 Cys in transmembrane domain. Which Cys could be responsible for potential disulfide bonds required for covalent dimer fomation (Lesley and Hyman, 1998)? One method would be to figure out firstly which domain of CD44 is required for dimer formation by analysis of truncated mutants, and then to mutate the Cys contained in this dimerization domain. Another alternative approach is to use fluorescence resonance energy transfer (FRET) which has been successfully used to identify the dimerization domain of receptor-like protein tyrosine phosphatase α (Jiang et al., 2000; Tertoolen et al., 2001). Two different GFP derivatives, for instance CFP and YFP, can be fused to the carboxyl terminus of CD44 in which the extracellular, transmembrane or cytoplasmic domain is truncated or mutated to construct two CD44 chimeras. By co-expressing these two chimeric CD44 proteins, it is possible to detect CD44 dimer in living cells by FRET analysis and to reveal dimerization domains.

4.3 The functional consequence of CD44 dimerization or oligomerization

Several cell adhesion molecules such as ICAM-1 and LFA-3 have all been shown to bind with higher affinity to their ligands when they are dimeric or multimeric (Detmers et al., 1987; Dustin et al., 1989). Aggregation and dimerization are also

important for binding of CD44 to HA (Lesley et al., 1993; Perschl et al., 1995). This can be concluded from several observations. A CD44-specific mAb, IRAWB14, induced HA binding when bound to CD44 as intact antibody or a multivalent Fab fragment, but not as a monovalent Fab, suggesting that clustering of the CD44 molecules is required for HA binding. Dimerization of CD44s was enforced by substituting the transmembrane domain of CD44 by that of the CD3ζ chain. The hybrid protein bound HA much stronger than wild type CD44 in intact cells. In my study binding to HA was observed in cells where CD44 dimers were detectable. Is CD44 dimer indeed decisive in mediating HA binding? To address this question directly, I used a conditional dimerization approach. I found that induced CD44 oligomerization played a modest role in HA binding. Consistent with my finding, CD44 oligomerization was found to be able to enhance HA binding ability to a similar extent in another T cell line (Lesley et al., 2000). Interestingly, by using the same dimerization system to study the oligomerization effect on ligand binding, other groups reported that the effect was modest (around 2 fold increment maximally) in the binding of PECAM-1 (Zhao and Newman, 2001), integrin $\alpha_{IIb}\beta_3$ (Hato et al., 1998) or cadherin (Yap et al., 1997) to their respective ligand.

4.4 CD44 in LB T cell lymphoma development

The invasion of LB cells into lymph node was blocked by anti-pan CD44 mAb, implicating that CD44 is required for LB lymphoma formation (Zahalka et al., 1995). LB cells express endogenous CD44s and little CD44 v4, v6 and v10 epitopes (Fig ?. and data not shown). Overexpression of CD44v4-10 facilitated local growth and metastasis of LB lymphoma in BALB/C mice (personal communication, Naor D.). I could conditionally regulate CD44 expression both *in vitro* and *in vivo* using the tet-off gene expression system. However, I found that CD44 variant isoforms failed to confer growth or metastatic advantage on LB lymphoma cells. Similarly, HA9 cells, which were obtained from LB cells through *in vitro* selection for HA binding and which express CD44 v4 and v6 epitopes, could not enhance tumor growth and invasiveness (Vogt Sionov and Naor, 1997). One explanation for these reports about

the contradictory roles of CD44 variant isoforms in cells of the same origin could be that LB cells underwent unknown genetic changes after passage in cell culture and therefore the results could differ when different LB cell sublines were used. *In vitro* studies showed much higher GAG binding of CD44 variant isoforms than CD44s. Since overexpression of CD44 variant isoforms did not promote lymphoma development, the GAG binding of CD44 might not be a decisive step for LB lymphoma growth and metastasis. Another finding was that tumor take rate was much lower in syngeneic mice challenged with LB transfectants than with LB parental cells, while injection into immunocompromised mice resulted in similar tumor take rates, suggesting that the immunogenic properties were changed in transfectants. An explanation could be that LB transfectants express high copies of tetracycline-controled transactivators which consist of a chimeric protein composed of Tet repressor sequences of *E. coli* and sequences of the VP16 protein of Herpes Simplex Virus (Gossen and Bujard, 1992; Gossen et al., 1995). Injection of the cells expressing this protein may cause immunological rejection.

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