Doctoral Thesis

In vivo gene targeting and in vitro proteolytic degradation; different approaches to study versican functions

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In Vivo Gene Targeting and in Vitro Proteolytic Degradation; Different Approaches to Study Versican Functions

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presented by

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Summary

Versican is a large chondroitin sulfate proteoglycan belonging to the hyalectans, a family of hyaluronan binding proteoglycans. In mammals, at least four versican isoforms (V0, V1, V2, V3) arising from alternative splicing, have been identified. During formation of the peripheral nervous system, the versican isoforms V0 and V1 are expressed in regions that flank or transiently block pathways of migratory neural crest cells and/or outgrowing axons. The expression of the smaller splice variant V2 is restricted to the extracellular matrix of the myelinated fiber tracts in the adult brain and spinal cord. Purified versicans V0, V1 and V2 strongly inhibit axonal growth in vitro. Recently, metalloproteases belonging to the ADAMTS family (a disintegrin and metalloproteinase with thrombospondin motif I) have been found to exhibit versicanase activity in in vitro assays. Versican fragments generated by ADAMTSs digestion correlate to those detected in human tissue extracts, suggesting a physiological role of ADAMTSs in the functional inactivation and degradation of versicans. These data support the hypothesis that versicans are involved in the development and stabilization of the nervous tissues by negatively modulating cell migration and axon outgrowth, whereas ADAMTSs may modulate versican activity during these processes. To further explore the putative inhibitory role of versicans we developed two complementary strategies: a) in vivo suppression of versican expression in mice and b) in vitro functional studies using versican proteolytically processed with ADAMTS endopeptidases.

To inhibit versican expression in mice we applied a conditional knockout approach, in which the versican promoter has been flanked by loxP sites. Two strains of mouse mutants carrying the loxP sites have been established in different genetic backgrounds. Mice with tissue specific inactivation of the four versican isoforms are currently being generated by crossing the loxP carrying strains with Cre-transgenic animals. In this way we should overcome the embryonic lethality caused by a defective heart development in the constitutive versican knockout mouse. Our results demonstrate that insertion of the loxP sequence flanking the versican promoter does not compromise the normal expression of versican.

In order to study the putative functional link between the expression of versicans and ADAMTS proteases, we performed a series of stripe choice assays comparing the inhibitory activities of full-length and ADAMTS-4-digested versican V2 on neurite outgrowth. Our experiments demonstrate that ADAMTS-4 mediated cleavage may partly reverse the axon growth inhibition. Since we could show that various ADAMTS endopeptidases with versicanase activity are expressed at critical time points during development, we speculate that ADAMTSs may also modulate the axonal growth suppressing function of versicans during the formation and maturation of the nervous system in vivo.

The here presented work forms now the basis to clarify the role of versicans and versican-degrading ADAMTS endopeptidases in various processes such as axon guidance, matrix stabilization and control of regeneration in the central and peripheral nervous system in vivo.
Versican è un condroitin solfato proteoglicano appartenente agli „hyalectani“, una famiglia di proteoglicani che si legano all’acido ialuronico. Nei mammiferi, sono state identificate almeno quattro isoforme (V0, V1, V2, V3) derivanti da splicing alternativo. Durante la formazione del sistema nervoso periferico, le isoforme V0 e V1 di versican sono espresse in regioni che fiancheggiano o bloccano in maniera transitoria le vie di migrazione delle cellule della cresta neurale e/o degli assoni in crescita. L’espressione di V2, la più piccola variante di splicing, è ristretta alla matrice extracellulare delle fibre nervose mielinate del cervello adulto e della spina dorsale. Versican V0, V1 e V2, purificati, inibiscono in maniera pronunciata la crescita degli assoni in vitro. Recentemente, si è riscontrato che delle metalloproteasi appartenenti alla famiglia delle ADAMTS (dall’inglese: a disintegrin and metalloproteinase with thrombospondin motif I) posseggono attività „versicanase“ in esperimenti in vitro. Frammenti di versican generati dalla digestione tramite ADAMTS coincidono a quelli detettti in estratti di tessuti umani, il quale suggerisce un ruolo fisiologico delle ADAMTS nell’inattivazione funzionale e nella degradazione di versican. Questi dati supportano l’ipotesi che i versicans siano coinvolti nello sviluppo e nella stabilizzazione del tessuto nervoso, modulando negativamente la migrazione delle cellule e la crescita degli assoni, mentre le ADAMTS potrebbero modulare l’attività dei versicans durante tali processi. Al fine di esplorare ulteriormente il ruolo inibitore putativo di versican, abbiamo sviluppato due strategie complementari: a) la soppressione in vivo dell’espressione di versican nei topi e b) studi funzionali in vitro utilizzando versican proteoliticamente processato tramite delle ADAMTS endopeptidasi.

Per inibire l’espressione di versican nei topi abbiamo applicato un approccio di „knockout“ condizionale, nel quale il promotore di versican è stato affiancato da sequenze loxP. Due razze di topi mutanti, portatori delle sequenze loxP, sono state generate in differenti ceppi genomici. Momentaneamente stiamo incrociando le razze portatrici dei loxP con animali transgenici per la Cre con lo scopo di ottenere topi nei quali le quattro isoforme di versican vengono inattivate in maniera specifica in differenti tessuti. In questa maniera dovremmo superare la letalità embrionica causata da uno sviluppo deficitario del cuore nel topo „knockout“ costitutivo per versican. I nostri risultati dimostrano che l’inserimento delle sequenze loxP fiancheggianti il promotore di versican, non compromettono la normale espressione di versican.

Al fine di studiare il collegamento funzionale putativo tra l’espressione di versican e quella delle proteasi ADAMTS, abbiamo effettuato un’estesa serie di esperimenti di scelta delle strisce tramite i quali abbiamo comparato l’attività inibitrice della crescita di neuriti di versican V2 intatto, e versican V2 digerito da ADAMTS-4. I nostri esperimenti dimostrano che la digestione mediata da ADAMTS-4 può invertire in maniera parziale l’inibizione della crescita degli assoni. Siccome abbiamo potuto dimostrare che le varie ADAMTS endopeptidasi con attività "versicanase" sono espresse durante momenti critici dello sviluppo, siamo portati a ritenere che le ADAMTS possano modulare la crescita degli assoni sopprimendo le funzioni di versican durante la formazione e maturazione del sistema nervoso in vivo. Il lavoro qui presentato, forma ora le basi per chiarire il ruolo di versican e delle ADAMTS endopeptidasi che degradano versican, in vari processi quali la guida degli assoni, la stabilizzazione della matrice e il controllo e rigenerazione del sistema nervoso centrale e periferico in vivo.
General Introduction

1. Proteoglycans

Proteoglycans are a group of glycoproteins that carry covalently linked glycosaminoglycan (GAG) side chains. GAGs consist of linear polysaccharides composed of approximately 20-200 disaccharide units. One of the two sugars residues in the repeating disaccharide is usually a uronic acid (glucuronic or iduronic, the second always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine). Depending on the composition of the disaccharide structure GAGs can be subdivided into: (1) hyaluronan, (2) chondroitin sulfate (CS) or dermatan sulfate (DS), (3) heparan sulfate (HS) and heparin, and (4) keratan sulfate (KS) (Figure 1). Hyaluronan is present on the cell surface and in the extracellular matrix as a free high molecular weight glycosaminoglycan. In contrast, all other GAGs are covalently linked to core proteins to form proteoglycans.

![Figure 1: Repeating disaccharide units of the different glycosaminoglycans (GAGs) (Bandtlow and Zimmermann, 2000).](image)

The large diversity of proteoglycans stems from the differences in the primary structure of the core proteins and in a large part from the individual variations in type, size and composition as well as distribution and substitution of the polysaccharide portion. Typical modifications of the GAG chains are: addition of O-sulfate groups, replacement of N-acetyl groups by N-sulfate and epimerization of D-glucuronic acid to L-iduronic acid. Further degrees of
structural complexity arise from N- and O-linked oligosaccharides and/or from the fact that more than one type of GAGs may be attached to the same core protein. Many proteoglycan core proteins are composed of multiple structural and functional domains as revealed by cDNA cloning and analysis of the deduced primary structures. Many of these domains may specifically interact with other molecules thus determining the biological activity of proteoglycans. Other modules may play an important role in recognition, sorting and delivery of proteoglycans to specific cellular locations including the extracellular matrix (ECM), the cell surface or within intracellular granules. This extensive structural diversity of proteoglycans is reflected in their diverse biological functions. These functions may be mediated by the GAG chains and/or the core proteins and include beside their structural roles also the storage and presentation of growth factors to cell surface receptors, assembly and organization of the extracellular matrix, modulation of axonal growth and cell-adhesion as well as regulation of tumor cell growth and invasion.

1.1. Biosynthesis of Proteoglycans

The biosynthesis of proteoglycans has been particularly well studied on the example of aggrecan (Schwartz, 2000), as it was considered for a long time to be the model proteoglycan. In general, the biosynthesis of the core proteins follows the common pattern of most membrane and secreted proteins: transcribed mRNA is translated in the cytosol and the nascent polypeptide translocated into the lumen of the ER via a signal sequence mediated process. After transport from the ER to the Golgi, the proteoglycans are then either secreted, transported to the cell membrane or to intracellular compartments. GAG synthesis takes place while the core protein is traversing the intracellular secretory pathway by adding one monosaccharide at a time at the end of the growing polysaccharide chain. Recently, molecular cloning of genes encoding for the various glycosyltransferases and sulfonyltransferases required for the GAGs chains synthesis has provided the basis to delineate the major biosynthetic pathways common to vertebrates and invertebrates (Prydz and Dalen, 2000). Heparan sulfate, chondroitin sulfate as well as dermatan sulfate are glycosidically attached on a common linkage tetrasaccharide that is covalently bound to specific serine residues. The formation of the tetrasaccharide linker is initiated by the enzyme xylosyltransferase (XylT), which catalyzes the addition of xylose from its nucleotide sugar precursor UDP-Xyl to the hydroxyl-group of a serine acceptor. This occurs during the transfer of the core protein from the rough endoplasmic reticulum to the cis Golgi. At present it is not completely clear what commits a protein to become a proteoglycan. The tetrapeptide Ser-Gly-Xaa-Gly (Xaa is any amino acid) preceded by acid amino acids has been proposed as consensus sequence for GAG chain initiation (Bourdon et al., 1987). However sequence analysis of the GAG attachment sites of different proteoglycans revealed that not all potential Ser-Gly sites are substituted, whereas also other sites containing Ser-Gly or Gly -Ser sequences may serve as xylose acceptor. Xylose addition may also be influenced by other factors such as proximity of substituted sites, downstream sequences or secondary structures (Sugahara and Kitagawa, 2000). Subsequent growth of the linkage region takes place in the early cis/medial Golgi by the sequential addition of two galactose residues catalyzed by distinct galactosyltransferases (GalT-I and GalT-II). Completion of the linkage oligosaccharide occurs in the medial/trans Golgi. This is mediated by the enzyme glucuronyltransferase I (GlcAT-I) which catalyze the addition of the initial glucuronic acid from UDP-glucuronic acid.
Core Protein

**Figure 2**: The linker tetrasaccharide.

After completion of the linker polysaccharide, the addition of the fifth saccharide determines whether the GAG chain becomes CS/DS or HS/heparin. Structural differences in the linkage region may determine the type and/or the character of the GAG species to be synthesized (Esko and Zhang, 1996).

N-acetylgalactosaminyltransferase I (GalNAcT-I) catalyzes the addition of GalNAc to the linkage tetrasaccharide leading to initiation of CS backbone elongation. Subsequent chain elongation requires two glycosyltransferase activities (GlcAT-II and GalNAcT-II) to add the alternating residues of glucuronic acid (from UDP-GlcA) and N-acetylgalactosamine (from UDP-GalNAc). Recently six glycosyltransferases potentially involved in the biosynthesis of CS/DS have been cloned and characterized (Figure 3). Among them, chondroitin synthase 1, 2, and 3 (CSS1, 2, and 3) exhibit both the GalNAcT-II and GlcAT-II activities required for the synthesis of the repeated di-saccharide units. The enzymes CSGalNAcT-I and CSGalNAcT-2 possess the GalNAcT-I activity that determine the initiation of CS synthesis as well as the GalNAcT-II activity involved in chain elongation. Finally, CSGlcAT has only the GlcAT-II activity, which is involved in chain elongation. The exact role of the different enzymes in the *in vivo* biosynthesis of chondroitin/dermatan sulfate GAGs has still to be elucidated.

Heparan sulfate (HS) chains are synthesized once GlcNAc is transferred to the common linkage region by the enzyme N-acetylgalactosaminyltransferase I (GalNAcT-I). This reaction is catalyzed *in vitro* by two members of the hereditary multiple exostoses gene family of tumor suppressors (EXTL 2 and EXTL 3).

Synthesis of the repeating region involves bifunctional enzymes with N-acetylgalactosaminyltransferase II (GalNAcT-II) and glucuronyltransferase II (GlcAT-II) activities. A hetero oligomeric complex formed by the enzymes EXT 1 and EXT 2 is probably responsible for the elongation of HS chains. The exact role of each protein in HS synthesis is still not clear since both enzymes have GalNAcT-II and GlcAT-II activities but deficiencies in either EXT 1 or EXT 2 cause defects in HS synthesis (McCormick et al., 2000). However also EXTL1 and EXTL3 posses the GlcNAcT-II activity needed for HS/Heparin chain elongation. This suggests that all the five EXT family members known to date are involved in the HS/Heparin synthesis with partially overlapping functions.
Chondroitin sulfate / Dermatan sulfate

The growing GAG chains are finally modified at various positions. The modifications include deacetylation / N-sulphation of GlcNAc units in HS and heparin, epimerization of GlcA and IdoA in HS, heparin and DS, and O-sulphation in various positions of the disaccharides of HS, heparin, CS and DS.

Keratan sulfates possess linkage regions that differ from the other GAGs. To date three different linkage structures have been identified leading to the designation of keratan sulfate I (KSI), keratan sulfate II (KSII) and keratan sulfate III (KSIII). In KSI the GAG chains are bound to Asn residues of the core protein via a complex-type N-linked oligosaccharide, whereas in KSII, GAGs are attached through an oligosaccharide structure that is linked O-glycosidically to the hydroxyl group of Ser or Thr. In the recently identified class KSIII, GAGs are attached to the core via a mannose-O-Ser linkage.

Extension of the KS chain occurs via the action of glycosyltransferases that alternatively add Gal and GlcNAc to the growing polymer. Despite the fact that several candidates displaying β-1,4-glucosyltransferase- or N-acetylgalcosaminyltransferase-activities have been described, it is still not clear which enzymes contribute to KS synthesis in vivo. O-sulphation of KS occurs at both the GalNAc and GlcNAc moieties. These modifications are made simultaneously with chain elongation and only take place at the growing of the polysaccharide. There is evidence that they are involved in the control of the length of the KS chains.

Finally, hyaluronan is the only GAG that is not covalently attached to a core protein and does not contain sulphate groups. It is synthesized at the plasma membrane and the nascent chain is secreted to the pericellular space. In contrast to the biosynthesis of chondroitin sulfate, it is elongated at the reducing end of the chain. Recently cDNA encoding three different hyaluronan synthases (HAS1, HAS2 and HAS3) have been cloned.
1.2. Nervous Tissue Proteoglycans

Proteoglycan expression was initially believed to be restricted to the extracellular matrix of cartilage. However, nowadays it has become clear that they are present in matrices of almost all tissues including brain and spinal cord. Early biochemical studies suggested that at least 25 different core proteins are present in the rat brain (Herndon and Lander, 1990). In recent years, the identification of cDNAs confirmed that a great variety of individual proteoglycans exists in the developing as well as in the mature central nervous system. Accurate characterization of their carbohydrate moiety has revealed that the majority of their core proteins carry either chondroitin sulfate or heparan sulfate side chains. They exist as extracellular matrix constituents (e.g. hyalectans), transmembrane components (e.g. syndecans) or are bound to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (e.g. glypicans).

Several lines of evidence suggest that the roles of proteoglycans in nervous tissues are much more complex than just providing structural support to cells and supplying tissue turgor. In the brain, proteoglycans colocalize with a variety of putative ligands including growth factors, cell adhesion molecules and matrix components, suggesting that proteoglycans modulate their functions. In-vitro studies have demonstrated that proteoglycans are actively involved in cellular processes like neuronal migration, axonal elongation and pathfinding as well as formation and stabilization of synapses. Since such processes are important for the accurate wiring of the nervous system it has been postulated that proteoglycans play an essential role during neuronal development as well as in adult brain activities by acting as modulators of cell-cell and cell-substrate interactions (Bandtlow and Zimmermann, 2000). In the following paragraphs, some of the nervous system proteoglycans will be described in more detail.

1.2.1. Syndecans

Syndecans belong to a family of transmembrane proteoglycans that carry predominantly HS side chains. In vertebrates four distinct genes have been characterized which code for: syndecan-1, syndecan-2 (also called fibroglycan), syndecan-3 (N-syndecan) and syndecan-4 (ryudocan, amphiglycan). Based on chromosomal localization, exon organization and sequence relationship with homologs found in Drosophila, Xenopus and C.elegans, mammalian syndecans are believed to be evolutionary ancient molecules that have arisen from a single ancestral gene by gene duplication and divergent evolution. All syndecans display a common structural organization including: a N-terminal signal sequence, an ectodomain carrying the GAG-attachment sites, a single hydrophobic transmembrane domain and finally a short C-terminal cytoplasmic domain. The ectodomain contains several Ser-Gly consensus sequences for GAG attachment. In syndecan-2 and -4 the GAG sites are localized at the N-terminal region, whereas in syndecan-1 and -3 they are clustered in two regions, one near the N-terminus and the other close to the plasma membrane. The syndecan ectodomain sequences are highly variable, the only exception being the GAG attachment sites. In contrast, the membrane spanning region and the cytoplasmic domain are greatly species conserved and show strong similarities among different syndecan family members, suggesting that these regions are important for specific interaction with membrane associated and cytoskeletal proteins. In detail, the cytoplasmic domain of each syndecan includes a first conserved stretch of 10 amino acids [RM(R/K)KKDEGSY] followed by a variable region unique to each family member. In all syndecan core proteins, it ends at the C-terminus with the conserved tetrapeptide sequence EFYA. Syndecans normally
bear covalently attached HS-GAGs, but under certain conditions CS-GAG can also be present. Despite the predicted, molecular size of the four core proteins between 20 and 40 kDa, they migrate on SDS-PAGE significantly slower, possibly due to core protein multimerization (Carey, 1997; Bernfield et al., 1999; Bandtlow and Zimmermann, 2000). Syndecans are believed to exert diverse functions. They may act as co-receptors for a number of extracellular matrix components including collagens, fibronectin, thrombospondin and tenascin. Consistent with this hypothesis, syndecan-1 localizes during early embryogenesis to the sites of matrix accumulation, whereas syndecan-4 is found in focal adhesion complexes. Recently, it was demonstrated that the EFYA sequence of the cytoplasmic tail of syndecans could bind to several PDZ proteins (Woods and Couchman, 1998). These findings suggest that upon binding to extracellular matrix components, syndecans may directly trigger intracellular signaling. Syndecans also act as co-receptors for a variety of heparin-binding growth factors including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), heparin binding epidermal growth factor (HB-EGF), vascular endothelial growth factor (VEGF).

The syndecan mediated signaling activity may be regulated by different mechanisms including the level of syndecan expression, ectodomain shedding and microdiversity of the glycosaminoglycan-chains. Besides growth factor and cell adhesion molecule binding, syndecans are known to interact with other extracellular ligands including pathogens (such as viruses, protozoa and bacteria) as well as enzymes and protease inhibitors. Thus, syndecans are also involved in cellular processes like blood coagulation, lipid metabolism and infection. Syndecans are expressed in a distinct cell-, tissue- and development-specific pattern, suggesting different functions for each family member. In general syndecan-1 is most abundant in epithelial tissue, syndecan-2 is mainly found in fibroblasts and syndecan-4 is expressed by multiple cell types, whereas expression of syndecan-3 is restricted to nervous tissues (Zimmermann and David, 1999).

Syndecan-2 is also present in the CNS where it is believed to play an important role in synaptogenesis. Syndecan-2 is expressed at the synapses at the time they reach their morphological maturation (Hsueh et al., 1998; Hsueh and Sheng, 1999). Furthermore overexpression of syndecan-2 in young hippocampal neurons accelerates the transformation of immature dendritic protrusions to mature dendritic spines receiving the synapses (Ethell and Yamaguchi, 1999).

In mouse brain the pattern of syndecan-3 expression correlates with the differentiation of oligodendrocytes and myelin formation. In neurons, binding of HB-GAM (heparin binding growth associated molecule) to syndecan-3 induces neurite outgrowth in which the cortactin-src kinase signaling pathway is involved (Kinnunen et al., 1996; Kinnunen et al., 1998). Furthermore, syndecan-3 may also play an important role in synaptic plasticity by regulating long-term potentiation (LTP) in CA1 region of the hippocampus (Lauri et al., 1998; Lauri et al., 1999).
1.2.2. Glypicans

The name glypican identifies a family of HS proteoglycans that are linked to the cell surface via GPI anchor. To date six different glypicans have been identified in mammals, two in Drosophila and one in C. elegans and Zebrafish (De Cat and David, 2001; Fransson, 2003). All glypican core proteins are approximately 60-70 kDa in size. They share a common domain organization including: a N-terminal signal sequence, a presumably compact globular domain characterized by the presence of 14 conserved cystein residues, a linker domain carrying the GAG-attachment sites and a hydrophobic C-terminal sequence needed for the attachment of the GPI anchor. Glypicans carry exclusively glycosaminoglycan chains of the HS type, their attachment sites being localized close to the plasma membrane. Depending on the particular core protein, glypicans may carry two to five GAG side chains. In addition, potential N- and O-glycosylation sites are present in glypican-1, -3, -4 and -5. Glypicans can be released to the extracellular space by the action of a phosphatidylinositol-specific phospholipase C or by proteolytic cleavage, as protease susceptible sites have been identified at the boundary between the globular and the linker domain in some family members. However, the significance of this processing is currently unknown. Based on sequence homology, glypican-1 and -2, glypican-3 and -5 and glypican-4 and -6 can be separated into three distinct subfamilies (Figure 5) (Bernfield et al., 1999).

In general, glypicans are expressed predominantly during development, with a specific spatio-temporal pattern suggesting an active involvement in morphogenesis (De Cat and David, 2001; Song and Poo, 2001). All family members are at least transiently expressed in neural tissues, glypican-2 (formerly called cerebroglycan) being restricted to the nervous system. In situ-hybridization analysis revealed a transient expression of glypican-2 in newly postmitotic neurons, which increases at the time of the final mitosis and disappears following migration and terminal differentiation (Stipp et al., 1994). Its localization is polarized to the actively growing axons especially in the growth cone (Ivins et al., 1997). Although these findings suggest a modulatory function in axonal growth and/or guidance, no obvious phenotype could be found in mice lacking glypican-2 expression. (Perrimon and Bernfield, 2000).
1.2.3. NG-2

NG-2 is a structurally unique single pass transmembrane proteoglycan expressed in central nervous tissues. Its core protein includes a large extracellular region, a single transmembrane polypeptide and a short cytoplasmic domain lacking homologies to known protein motifs. The extracellular portion consists of two cysteines containing domains, which are separated by a 950 amino acid region to which two to three CS chains can be attached. The 76 amino acids long intracellular tail has been recently demonstrated to bind the seventh PDZ domain of the glutamate receptor interacting protein-1 GRIP1. This interaction results in clustering of NG-2 and AMPA glutamate receptors that may contribute to glial neuronal signaling (Stegmuller et al., 2003).

In the central nervous system NG-2 is expressed on the surface of oligodendrocyte precursor cells of the O2A lineage (Levine and Nishiyama, 1996). Purified NG-2 inhibits axonal growth in vitro (Dou and Levine, 1994; Chen et al., 2002), the inhibitory activity being localized in the N-terminal region and the juxtamembrane globular elements of the ectodomain (Ughrin et al., 2003). Several studies demonstrated that NG-2 expression is upregulated after CNS injury in cells that compose the glial scar, suggesting that NG-2 interferes with axonal regeneration (Levine, 1994; Nishiyama et al., 1996). However NG-2 knockout mice do not exhibit any obvious phenotype (Grako et al., 1999).

1.2.4. RPTPβ/Phosphacan

The receptor-type protein-tyrosine phosphatase β (RPTPβ) is another transmembrane proteoglycan expressed in the nervous system. Three isoforms have been identified, which arise from alternative mRNA splicing. The largest full-length RPTPβ isoform contains a carbonic anhydrase domain located closely to the N-terminus of the extracellular domain. It is followed by a fibronectin type IIIrepeat and a cystein free stretch, which carries the GAG
chains. Finally, two intracellular tyrosine phosphatase domains are localized c-terminally to the single transmembrane-spanning region. The second membrane bound RPTPβ splice-variant lacks 860 amino acids of the ectodomain including most of the GAG attachment sites. The third isoform containing the extracellular portion terminating shortly before the transmembrane domain has been named phosphacan. It exists as a secreted proteoglycan carrying either exclusively CS or CS and KS GAG chains (phosphacan-KS).

Expression analysis revealed that RTPTβ/phosphacan is predominantly expressed along radial glial fibers and on migrating neurons (Canoll et al., 1993; Maeda et al., 1995). Furthermore the extracellular domain of RTPTβ/phosphacan binds various cell adhesion and extracellular matrix molecules like N-CAM, Ng-CAM, Nr-CAM, contactin and tenascins as well as pleiotrophin, a secreted heparin binding protein that stimulates neurite outgrowth *in vitro*. Taken together all these findings suggest that RTPTβ/phosphacan may modulate neuronal migration (Maeda and Noda, 1998).

1.2.5. Hyalectans

Hyalectans (or lecticans), form a family of proteoglycans that carry mainly chondroitin sulfate chains. Four hyalectans have been identified in mammals, namely versican (Zimmermann and Ruoslahti, 1989), aggrecan (Doege et al., 1991), neurocan (Rauch et al., 1992) and brevican (Yamada et al., 1994). Dermacan, a structurally related but yet distinct core protein, has recently been characterized in zebrafish (Kang et al., 2004). The name "hyalectan" has been deduced from some structural and functional features common to all family members: 1) the ability to bind hyaluronan (G1 domain), and 2) the presence of a C-type lectin-like element located in the C-terminal globular structure (G3 domain). The G1 and G3 domains of versican, aggrecan, neurocan and brevican are highly conserved and mediate interactions with hyaluronan and tenascins, respectively. In contrast, the central domains of hyalectans, which contain the attachment sites for chondroitin sulfate chains, are highly diverse in terms of size and sequence. The N-terminal globular domain of each hyalectan consists of an immunoglobulin- (Ig) like domain followed by a hyaluronan-binding tandem repeat. In aggrecan an additional globular domain is present that contains a second tandem repeat without the Ig-like element (G2). The specific function of this domain is presently unclear. The C-terminal globular domain includes one or two epidermal growth factor- (EGF) like repeats, a C-type lectin-like domain and a sushi- (or complement regulatory protein, CRP-) element.

All four hyalectans are expressed in nervous tissues at certain stages, however each hyalectan has a characteristic spatio-temporal distribution pattern during development and in adulthood, possibly playing different roles in the extracellular matrix of the nervous system.
1.2.5.1. Aggrecan

Aggrecan was first characterized as a large aggregating cartilage proteoglycan. In the extracellular matrix of cartilage it interacts with hyaluronan and link protein to form high molecular aggregates. A single aggregate may reach a length of up to 15 µm and cover an area roughly equivalent to the size of an entire cell. The 250 kDa aggrecan core protein typically carries around 100 CS chains, 30 KS chains and several N- and O- linked oligosaccharides. Consequently, the effective molecular weight of each aggrecan molecule is in the range of $3 \times 10^6$ Da, with the carbohydrate moiety contributing roughly 90% of the mass. Due to the high number of negatively charged GAG chains aggrecan retains large amounts of water giving cartilage its unique gel-like property and resistance to compression.

In addition to cartilage tissues, aggrecan is also expressed in the developing as well as the mature brain. Whereas the core protein of the cartilage- and brain-aggrecan are the product of a single gene, tissue-specific differences due to posttranslational processing are eminent. In fact, brain-derived aggrecan carries significantly fewer and shorter CS chains and lacks keratan sulfate (Li et al., 1996).

During rat brain development the concentration of aggrecan increases steadily and reaches a plateau at about 150 days post-natal, leading to the speculation that aggrecan may be involved in the mechanism that arrests neural migration (Schwartz et al., 1996). Experiments with purified non neuronal aggrecan demonstrated its inhibitory activity in the extension of axons from retinal and sensory neurons as well as in the migration of neural crest cells, suggesting a modulatory role of aggrecan in migration processes during development of the nervous system. Yet, these functional data still await confirmation in experiments with brain-derived aggrecan.

Naturally occurring mutants that carry a defective aggrecan gene have been described in mice (cmd strain, cartilage matrix deficiency) and chicken (nm, nanomelia). The mutations lead in both species to premature termination of the aggrecan protein translation. These strains display severe abnormalities in skeletal development, but no neuronal aberrations have been identified so far (Velleman and Clark, 1992; Watanabe et al., 1997).
1.2.5.2. Brevican

Brevican is a brain specific proteoglycan that is expressed in myelinated fiber tracts, around cerebellar glomeruli and in the perineuronal nets present around large neurons throughout the brain (Bruckner et al., 2000). Alternative splicing gives rise to two isoforms; a secreted full-length protein and a shorter GPI-linked. Brevican is considered a “part time” proteoglycan, as it exists either as a CSPG, carrying three CS chains, or in a GAG-free form. Proteolytic cleavage, occurring mainly in the adult brain, gives rise to the separation of the N-terminal region from the C-terminal part of the molecule. Interestingly, brevican cleavage has been correlated with an invasive phenotype of glioma tumors (Matthews et al., 2000). Brevican expression in the mouse starts at embryonic day 14, increases significantly post-natal and reaches a plateau at around 150 days after birth. In vitro assays demonstrate that brevican is inhibitory to cell adhesion and neurite outgrowth and that the inhibitory activity resides in the carbohydrate moiety (Yamada et al., 1997). Brevican deficient mice showed impairment in hippocampal CA1 long-term potentiation but no obvious deficits were seen in behavioral tasks focusing on learning and memory (Brakebusch et al., 2002).

1.2.5.3. Neurocan

During development, neurocan is a major component of the brain extracellular matrix being predominantly expressed by neurons. The neurocan core protein has a predicted molecular weight of 136 kDa and is substituted with about three CS chains, 5 to 6 N-linked and up to 40 O-linked oligosaccharides. During rat brain development the CS chains increase in size and a shift from 6 to 4-O-sulphation occurs. At the end of neurogenesis an increasing fraction of neurocan is proteolytically processed, resulting in two fragments of about 130 and 150 kDa. In the rat brain, neurocan expression is first detected at embryonic day 12. It increases during late embryogenesis but decreases significantly during the first month after birth (Milev et al., 1998). In in vitro assays neurocan inhibits neurite outgrowth and cell adhesion, presumably interfering with NCAM and NgCAM homophilic binding (Li et al., 2000). Furthermore neurocan accumulates in certain brain regions that are avoided by axons and in the glial scar formed after brain injury (Katoh-Semba et al., 1995; Watanabe et al., 1995; Haas et al., 1999; Asher et al., 2000). Taken together these data suggest that during development neurocan modulates axonal outgrowth and pathfinding whereas in the case of CNS injuries it may interfere with axonal regeneration. Interestingly mice that lack neurocan expression do not show any obvious phenotype (Zhou et al., 2001).

1.2.5.4. Versican

1.2.5.4.1. Versican Gene Structure and Isoforms

The versican gene (Cspg2) has been localized on chromosome 13 in the mouse (Naso et al., 1995) and on chromosome 5q14.3 in the human genome (Iozzo et al., 1992). The gene is subdivided into 15 exons and extends over 90-100 kb (Shinomura et al., 1995). Similar to the other hyalectans the organization of the versican gene shows a remarkable conservation
between the exon-intron architecture and the domain structure of the core protein. Exon I of the versican gene contains part of the 5'-untranslated sequence. The remainder of the 5'-UTR is located in exon II, which also includes the translation start codon. The Ig-like loop is encoded by the exon III, while the tandem repeats which are responsible for the binding to hyaluronan are located in exon IV, V and VI. Exon VII and VIII are very large in size and encode the two different chondroitin sulfate attachment domains named GAG-α and GAG-β, respectively. The two EGF-like tandem repeats are encoded by exon IX and X, while the C-type lectin-like motif is located in exons XI, XII, and XIII. Exon XIV encodes for the sushi element and exon XV contains the rest of the C-terminal coding region and the whole 3'-untranslated region.

In spite of the presence of only one versican gene, several versican core proteins have been identified (Dours-Zimmermann and Zimmermann, 1994; Ito et al., 1995; Zako et al., 1995). This structural diversity arises from alternative splicing of mRNA generating at least four different splice variants in mammals.

In men and mice the difference between the isoforms results from the alternative usage of the two giant exons VII and VIII of 3 kb and 5.3 kb size in the human and 2.9 and 5.2 kb length in the mouse gene. These two exons encode for the central glycosaminoglycan carrying domains, termed GAG-α and GAG-β, respectively. The largest V0 splice variant, of about 12 kb, carries both the GAG-α and GAG-β domains and includes between 17 and 23 putative chondroitin sulfate attachment sites. The smaller V1 and V2 isoforms, of about 9 kb and 6.5 kb, only contain either the GAG-β or GAG-α domain, respectively. Thus the number of the putative chondroitin sulfate chains is reduced to 12-15 in V1 and to 5-8 in V2 (Dours-Zimmermann and Zimmermann, 1994). The fourth versican isoform V3 is composed of only the N- and C-terminal globular domains. Because of the lack of all potential chondroitin sulfate attachment sites, the V3 isoform appears not to be a proteoglycan, but may exist as a glycoprotein (Zako et al., 1995).

Figure 7: Schematic representation of the versican gene showing the correlation between the exon/intron organization and the domain structure. UTR: untranslated region, SP: secretory signal sequence, ATG: transcription start codon, Ig: immunoglobulin-like loop, HABR: hyaluronan-binding repeats, GAG: glycosaminoglycan-attachment domain, EGF: epidermal growth factor-like element, Lectin: C-type lectin-like domain, Sushi: sushi module.

Figure 8: The four versican splice variants generated by the alternative usage of the exons VII and VIII respectively.
The expression of the versican gene is controlled by a promoter that harbors a typical TATA box and putative binding sites for several transcription factors potentially implicated in the tissue specific expression of versican (Naso et al., 1994). Deletion analysis of the human versican promoter revealed a cluster of AP2-binding sites upstream of the TATA box that may act as an enhancer element. Also present in the human versican promoter are putative binding sites for xenobiotic responsive element (XRE)-binding factor, CCAAT binding transcription factor, SP1, CCAAT enhancer-binding protein, and cyclic AMP-responsive element-binding protein. Several of those binding sites are conserved between the human and the mouse versican promoter. Hence, together with additional cis-acting elements in the first intron (Yoon et al., 2002) they are likely to regulate versican expression in vivo.

Three potential transcription initiation sites, located 313 (site I), 280 (site II) and 254 (site III) bases upstream from the 3' end of the first exon were postulated based on the results of a modified 5'-RACE analysis (Shinomura et al., 1995). The TATA box is located 28 bases upstream from site II, while no TATA consensus sequence is present in the vicinities of site I and site III. Whether these two sites are indeed active remains to be shown in further experiments.

1.2.5.4.2. Versican Expression

Using immunohistochemical and RT-PCR techniques, versican was detected in a number of morphologically active tissues during embryogenesis (Kimata et al., 1986; Shinomura et al., 1990; Yamagata et al., 1993a; Landolt et al., 1995). For instance, versicans V0/V1 are transiently expressed during early development in tissues that act as non permissive barriers to neural crest cell migration from the central neural tube and to the outgrowth of axons from the dorsal root ganglions and the motor columns (Landolt et al., 1995). At later stages the expression of versican V0/V1 is restricted to the pre-chondrogenic mesenchyme and the developing dermis. In adult tissue the expression of versican V0/V1 is limited to blood vessels and to the loose connective tissues of various organs. To a lesser extent, these isoforms are also present in fibrous and elastic cartilage, in tendon and skeletal muscle and in the dermis and the basal layer of the epidermis (Zimmermann et al., 1994; Bode-Lesniewska et al., 1996). A transient expression of versican V0/V1 is detected in the central nervous system during late embryonic and early postnatal brain development, being replaced in the adult brain by the V2 isoform (Milev et al., 1998; Schmalfeldt et al., 1998; Schmalfeldt et al., 2000). The expression of this smaller isoform, which is a major component of the adult brain tissue, appears to be restricted to the central nervous system of the adult organism (Dours-Zimmermann and Zimmermann, 1994).

1.2.5.4.3. Versican Ligands

Versican interacts with a number of extracellular and cell surface ligands. Interactions may be mediated by specific core protein domains or by the chondroitin sulfate chains, the latter being relatively little characterized. Like the other lecticans, versican binds through its N-terminal globular domain to hyaluronan. In in vitro reconstitution studies it was shown that this interaction is stabilized by members of the link protein family, which bind simultaneously to versican and hyaluronan molecules. Recently it was found that the brain specific link protein (Bral1) co-localizes with Versican V2 at the node of Ranvier, suggesting that the hyaluronan/versican/link protein ternary complex may also exist in vivo (Oohashi et al., 2002). Early studies using recombinant core protein fragments showed that the C-type lectin
domain of versican binds simple carbohydrates including D-mannose, D-galactose, L-fucose, N-acetylglucosamine, heparin and heparan sulfate (Ujita et al., 1994) and sulphated glycolipids (Miura et al., 1999) in a calcium dependant manner. Whereas these findings suggests that versican may interact with a number of glycoproteins and lipid components of the cell membrane the physiological significance of these protein-carbohydrate-interactions are still not understood. Conversely, several candidate ligands for protein-protein-mediated interactions with the C-terminal globular domain of versican have been identified recently. These include members of the tenascin (Aspberg et al., 1995; Aspberg et al., 1997; Rauch et al., 1997) and the fibulin family (Aspberg et al., 1999; Olin et al., 2001). In particular tenascin-R and potentially also tenascin-C bind through their fibronectin type III repeats 3-5 to the C-type lectin domain of versican. Tenascin-R is specifically expressed in the central nervous system were it co-localizes with versican V2 at the node of Ranvier (Dours-Zimmermann et al. unpublished data). Fibulin-1 and fibulin-2 bind to the lectin domain of versican through their EGF-like repeats in domain II and colocalize with versican in skin and aorta.

Figure 9: Rotary shadowing electronmicrograph of aggregates of aggregcan, cartilage link protein and hyaluronan in cartilage extracts. Analogous complexes of versican V2 hyaluronan and Bral-1 are likely present in the central nervous system.

1.2.5.4.4 Putative Functions

Besides the common function of proteoglycans in structurally supporting the extracellular matrices, versican may be involved in multiple biological processes. Studies to date suggest an active implication of versican in the control of cell adhesion, migration, proliferation and neurite outgrowth. Versican has been shown to interfere with the attachment of embryonic fibroblasts to various extracellular substances including fibronectin, laminin and collagen (Yamagata et al., 1989). Furthermore versican is completely excluded from focal contact sites of cultured fibroblasts and fails to support the adhesion of chicken neural crest cells (Yamagata et al., 1993b; Perris et al., 1996). These notions correlate with the finding that versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell
migration and axonal outgrowth, suggesting that versican may antagonize cell adhesion and thus regulate cell and axonal migration (Landolt et al., 1995). This hypothesis has recently been supported by the finding that isolated versican V2 (Schmalfeldt et al., 2000) and also the larger isoforms V0 and V1 (S. Raghav-Dutt, manuscript in preparation) inhibit the outgrowth of neurites from retinal and dorsal root ganglion cells in vitro. Furthermore, versican V2 has been identified as one of the major axon growth inhibitory components in myelin preparations of the central nervous system being expressed by cells of oligodendrocyte lineage (Niederöst et al., 1999). The timely upregulation of the V2 isoform during central nervous system maturation correlates with the reduced plasticity and loss of regenerative capacity of brain and spinal cord.

1.3. Catabolism of Proteoglycans

The extended structure of proteoglycans renders them particularly susceptible to an attack by several types of degradative agents, both enzymatic and non-enzymatic in nature. Various classes of proteinases have been shown to cleave the core proteins, whereas sulfatases and glycosidases degrade the GAG side chains. In addition free radicals can induce polymer deterioration of both the protein and carbohydrate components. Usually, degradation of extracellular proteoglycans is initiated by secreted proteinases that cleave the core protein. Complete degradation occurs intracellularly within the lysosomes, where multiple proteinases, sulphatases and glycosylases convert both the protein and polysaccharide components into their constituent units. Careful control of proteoglycan catabolism is an important physiological process. For example, coordinated spatio-temporal matrix proteolysis is crucial for proper tissue growth and remodeling during embryonic development. In contrast uncontrolled proteoglycans degradation play important roles in several pathological conditions like arthritis, tumor invasion and metastasis.

A number of enzymes have been implicated in proteoglycan catabolism including members of the four main classes (aspartyl-, serine-, cysteine- and metallo-proteinases). However current data indicate that initiation of proteoglycans breakdown is largely controlled by the extracellular action of metalloproteinases. The main structural feature of this class of enzymes is the presence of a metal ion (almost exclusively Zn$^{2+}$) in the active site of the catalytic domain. In particular the members of three subfamilies of the metalloproteinases, the matrix metalloproteinases (MMP), the ADAMs (a disintegrin and metalloprotease) and the ADAMTSs (a disintegrin and metalloprotease with thrombospondin type I motif) are believed to be the major mediators of extracellular proteoglycan degradation.

1.3.1. The Matrix Metalloproteinases

The MMPs are a family of zinc-dependent proteinases, which are collectively capable of degrading virtually all components of the basement membrane and extracellular matrix. The members of the MMP family are structurally highly conserved, possess similar functional features and follow analogous mechanism of proteolysis. In human 24 different MMPs have been identified, that can be loosely subdivided on the basis of their structural organization in: 9 archetypal MMPs, 2 matrilysins, 2 gelatinases, and 10 convertase-activatable MMPs (Figure 10).

The archetypal MMPs consist of a signal peptide necessary for secretion, an N-terminal propeptide domain that keeps the enzyme in an inactive state, a central catalytic domain
containing the conserved amino acid sequence (HEXGHXXGXXH, where X is any amino acid) that coordinates the catalytic zinc ion, and a hemopexin C-terminal domain that confers substrate specificity.

Matrylilins also called minimal domain MMPs, lack the hemopexin domain. Gelatinases differ from the other MMP in that they have three fibronectin type II repeats in the catalytic domain that mediate binding to gelatin. Finally convertase-activable MMPs are characterized by the presence of a basic insert in the propeptide that is cleaved intracellularly by furin-like proteases.

Most of the MMPs are secreted into the extracellular space, although some are anchored to the cell surface by a transmembrane segment (MMP-14, -15, -16 and -24) or a glycosylphosphatidylinositol (GPI) anchor (MMP-17, and -25).

**Figure 10: The MMPs family.**

The regulation of MMPs occurs at least on three levels: transcription, proteolytic activation of the zymogen form and inhibition of the active enzyme through inhibitory molecules. Under normal conditions MMP expression is minimal, whereas under specific physiological circumstances like embryogenesis, wound repair, inflammation or oncogenesis their expression is rapidly induced. Numerous cytokines, growth factors and ECM components as well as physical cellular interactions have been involved in the up-regulation of MMP expression (Stamenkovic, 2000).

Like other proteolytic enzymes MMPs are first synthesized as inactive proenzymes orzymogens. Activation of the secreted MMP proforms occurs through cleavage of the N-terminal prodomain either via the plasminogen cascade system (Atkinson et al., 1995) or by other already activated MMPs (Fridman et al., 1992). The convertase activatable MMPs represent an exception, as they are activated within the Golgi apparatus by a furin-like convertase (Nagase and Woessner, 1999).

The activated forms of MMPs are subjected to inhibition by several natural molecules. The major physiological inhibitors of the MMPs belong to a family of proteins known as the tissue
inhibitors of metalloproteinases (TIMP). To date four different TIMPs have been described, which bind in a reversible way to the MMPs in a 1:1 ratio. Other molecules involved in the regulation of MMP activity include: α2-macroglobulin, thrombospondin and the cell surface receptor RECK (reversion-inducing cystein-rich protein with kazal motifs) (Stamenkovic, 2003).

MMPs were initially described as proteases able to degrade ECM components including proteoglycans. Thus their main function was believed to be the remodeling of the ECM for tissue maintenance or to facilitate migration. More recently it has been demonstrated that MMP substrates also include non-matrix molecules like growth factor precursors and binding proteins to cell surface adhesion receptors (Table 1). It is now known that MMPs also regulate several physiological and pathological events like: tissue morphogenesis, wound healing, cell migration, proliferation and apoptosis as well as tumor invasion and metastasis. The mechanisms by which MMP activity may modulate those events include: 1) degradation of the ECM molecules to allow cell migration, 2) cleavage of matrix proteins resulting in the release of bioactive molecules that were sequestered in the ECM, 3) direct cleavage and activation of non-matrix substrates.

Table 1: Human matrix metalloproteinases and their substrates

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Alternative name</th>
<th>Collagenous substrates</th>
<th>Non-collagenous ECM substrates</th>
<th>Other substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>collagenase-1</td>
<td>collagen types I, II, III, VII, VIII, X, and gelatin</td>
<td>aggrecan, casein, nidogen, serpins, versican, perlecan, proteoglycan link protein 1, and tenasin-C</td>
<td>α1-antichymotrypsin, α1-antitrypsin/α1-proteinase inhibitor, IGFBP-3, IGFBP-5, IL-1β, L-selectin, ovostatin, recombinant TNF-α peptide, and SDF-I</td>
</tr>
<tr>
<td>MMP-2</td>
<td>gelatinase-A</td>
<td>collagen types I, IV, V, VII, X, XI, XIV, and gelatin</td>
<td>aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein 1, and versican</td>
<td>active MMP-9, active MMP-13, FGF RI, IGFBP3, IGFBP5, IL-1β, recombinant TNF-α peptide, and TGF-β</td>
</tr>
<tr>
<td>MMP-3</td>
<td>stromelysin-1</td>
<td>collagen types II, IV, IX, X, and gelatin</td>
<td>aggrecan, casein, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan link protein 1, and versican</td>
<td>α1-antichymotrypsin, α1-proteinase inhibitor, antithrombin III, E-cadherin, fibrinogen, IGFBP-3, L-selectin, ovostatin, pro-HB-EGF, pro-IL-β, pro-MMP-1, pro-MMP-8, pro-MMP-9, pro-TNFα, and SDF-I</td>
</tr>
<tr>
<td>MMP-7</td>
<td>matrilysin-1, neutrophil collagenase</td>
<td>collagen types I, II, III, IV, V, and X</td>
<td>aggrecan, casein, elastin, enactin, laminin, and proteoglycan link protein</td>
<td>β4 integrin, decorin, defensin, E-cadherin, FAS-L, plasminogen, pro-MMP2, pro-MMP7, pro-TNFα, transferrin, and syndecan</td>
</tr>
<tr>
<td>MMP-8</td>
<td>collagenase-2</td>
<td>collagen types I, II, III, V, VII, VIII, X, and gelatin</td>
<td>aggrecan, laminin, and nidogen</td>
<td>α2-antiplasmin, and pro-MMP8</td>
</tr>
<tr>
<td>MMP-9</td>
<td>gelatinase-B</td>
<td>collagen types IV, V, VII, X, and XIV</td>
<td>fibronectin, laminin, nidogen, proteoglycan link protein, and versican</td>
<td>CXCL5, IL-1β, IL-2-R, plasminogen, pro-TNFα, SDF-I, and TGF-β</td>
</tr>
<tr>
<td>MMP-10</td>
<td>stromelysin-2</td>
<td>collagen types III, IV, V, and gelatin</td>
<td>fibronectin, laminin, and nidogen</td>
<td>pro-MMP-1, pro-MMP-8, and pro-MMP10</td>
</tr>
<tr>
<td>MMP-11</td>
<td>stromelysin-3</td>
<td>laminin</td>
<td>α1-antitrypsin, α1-proteinase inhibitor, and IGFBP-1</td>
<td></td>
</tr>
</tbody>
</table>
MMP-12 macrophage metalloelastase collagen types I, II, III, IV, V, IX, X, XI, and gelatin elastin aggrecan, fibronectin, laminin, perlecarn and tenascin plasminogen activator 2, pro-MMP-9, pro-MMP-13, and SDF-I

MMP-13 collagenase-3 collagen types I, II, III, and gelatin aggrecan, dermatan sulphate proteoglycan, fibrin, fibronectin, laminin, nidogen, perlecarn, tenascin, and vitronectin α,β integrin, CD44, gC1qR, pro-MMP-2, pro-MMP-13, pro-TNFα, SDF-I, and tissue transglutaminase

MMP-14 MT1-MMP collagen types I, II, III, and gelatin aggrecan, fibronectin, laminin, nidogen, perlecarn, tenascin, and vitronectin pro-MMP-2, pro-MMP-13, and tissue transglutaminase

MMP-15 MT2-MMP collagen types I, II, III, and gelatin aggrecan, fibronectin, laminin, nidogen, perlecarn, tenascin, and vitronectin pro-MMP-2, and pro-MMP-13

MMP-16 MT3-MMP collagen types I, III, and gelatin aggrecan, casein, fibronectin, laminin, perlecarn, and vitronectin pro-MMP-2 and pro-MMP-13

MMP-17 MT4-MMP gelatin fibrin, and fibronectin

MMP-19 RASI-1 collagen types I, IV and gelatin aggrecan, casein, fibronectin, laminin, nidogen, and tenascin

MMP-20 enamelysin aggrecan, amelogenin, and cartilage oligomeric protein

MMP-21 X-MMP casein, fibrinogen, and fibronectin α1-antitrypsin

MMP-22 CA-MMP gelatin

MMP-23 MT5-MMP gelatin chondroitin/dermatan sulfate proteoglycans and fibronectin pro-MMP-2, and pro-MMP-13

MMP-25 leukolysin, MT6-MMP collagen type IV and gelatin fibrin and fibronectin pro-MMP-2

MMP-26 matrilysin-2, endometase collagen types IV, and gelatin casein, fibrinogen, and fibronectin β1-proteinase inhibitor

MMP-27 MMP-22, C-MMP casein

MMP-28 epilysin casein

(*) Although there are 24 human MMPs, 29 numbers have been used in the literature. The symbols MMP-4, MMP-5, MMP-6 and MMP-29 are redundant in humans and are no longer in use; MMP-18 corresponds to a *Xenopus laevis* collagenase, for which no human ortholog is known, and a human protein published as MMP-18 is now called MMP-19. Two nearly identical genes found in a segment of chromosome 1 that is duplicated were called MMP-21 and MMP-22 but are now referred to as MMP-23A and MMP-23B.

1.3.2. The ADAMTS

The ADAMTS (a disintegrin and metalloprotease with thrombospondin type I motif) form a family of secreted proteinases related to the transmembrane ADAMs (Tang and Hong, 1999; Kaushal and Shah, 2000; Tang, 2001). The members of the only recently discovered ADAMTS family exhibit a highly conserved domain organization, including an N-terminal signal peptide for targeting to the secretory pathway, a prodomain, a catalytic (protease) domain, a disintegrin-like domain for adhesion and a thrombospondin 1-like motif (TSP1). This is followed by a cystein rich element, a spacer domain and finally a variable number of C-terminal TSP motifs. Several additional C-terminal domains have been found downstream of the variable TSP1 repeats in some ADAMTS molecules. These include: a mucin domain, a CUB domain (a 110-115 amino acid domain present in certain developmentally regulated
proteins, and named after the first three identified proteins containing these domains, complement subcomponent Clr/Cls, Uegf and bone morphogenic protein 1), and a PLAC domain (a 30-40 amino acid peptide containing six cysteines, first identified in the moth protein lacunin). ADAMTSs differ from ADAMs not only by the presence of a variable number of TSP repeats but also by the lack of a transmembrane domain. They are therefore secreted proteins that associate with the ECM or bind to the cell surface by interacting with membrane bound proteins. The presence of a Zn$^{2+}$-binding consensus sequence (HEXXHXXNXXH) in all ADAMTS family members that is highly similar to the catalytic site found in several MMP proteases, suggests that all ADAMTS currently known are catalytically active.

ADAMTS activation occurs in the trans Golgi by furin mediated cleavage of the N-terminal prodomain, thus ADAMTS are secreted as potentially active proteinases. As reported for ADAMTS-4 (Flannery et al., 2002; Gao et al., 2002; Kashiwagi et al., 2003), additional C-terminal processing, mediated by autocatalysis or MMPs, may modulate enzyme activity and substrate specificity.

Endogenous inhibitors of ADAMTS activity, such as members of the TIMP family (Hashimoto et al., 2001) or the general endoproteinase inhibitor α2 macroglobulin (Tortorella et al., 2004), may also be involved in the modulation of enzyme activity. In general TIMP-3 appears to be the most efficient inhibitor of ADAMTS, with submolar Ki for ADAMTS-4 and -5 (Kashiwagi et al., 2001), whereas the other TIMPs have only weak effects on ADAMTS activity. TIMPs can also indirectly modulate ADAMTS activation by inhibiting the MMPs involved in C-terminal truncation. This mechanism was recently described by Gao et al., who demonstrated that the generation of active C-truncated ADAMTS-4 forms is in human chondrosarcoma cells promoted by cotransfection with MT-MMP14. This activation is completely blocked by the MT-MMP14 inhibitor, TIMP-1 (Gao et al., 2003).

To date at least eighteen human ADAMTS have been reported (Somerville et al., 2003). Most of them have been characterized only at the structural level and their biological functions remain to be identified. Few ADAMTSs have been recombinantly expressed, purified and tested for proteolytic activities and biological functions. In general ADAMTSs exhibit a more restricted substrate specificity than MMPs. For example ADAMTS-2, -3 and -14 are specific procollagen N-proteinases responsible for the processing of amino-propeptide of fibrillar collagens (Colige et al., 1997; Colige et al., 2001; Fernandes et al., 2001). Mutations in ADAMTS-2 causes the recessively inherited connective tissue disorder dermatosparaxis in animals and the Ehlers-Danlos syndrome-VIIC in humans (Colige et al., 1999), disorders characterized by severe fragility of the skin. A similar phenotype was also observed for ADAMTS-2 null mice (Li et al., 2001). ADAMTS-13 was identified as a von Willebrand Factor-cleaving proteinase. Mutations in the gene encoding this enzyme cause the inherited thrombotic thrombocytopenic purpura, a coagulation disorder due to the deficient proteolysis of von Willebrand factor (Levy et al., 2001). Several ADAMTS have been reported to degrade proteoglycans of the hyalcan family with a certain degree of specificity. ADAMTS-4, -5 and less efficiently ADAMTS-1 cleave aggrecan at different sites, and are probably involved in cartilage degradation during arthritis (Abbaszade et al., 1999; Tortorella et al., 1999; Kuno et al., 2000). ADAMTS-4 is also responsible for brevican cleavage (Nakamura et al., 2000), a process that correlates in gliomas with increased invasiveness (Matthews et al., 2000). Furthermore a link between ADAMTS-4 overexpression and brevican proteolysis was reported in kainate-induced CNS lesions (Yuan et al., 2002). Finally ADAMTS-1, -4, -9 show versicanase activity in vitro (Sandy et al., 2001; Somerville et al., 2003; Westling et al., 2004), whereas to date no ADAMTS was reported to cleave neurocan, the fourth member of the hyalcan family.
1.3.2.1. Hyalectans Degradation

All hyalectans have been shown to undergo proteolytic cleavage of the core proteins. This may contribute to the normal hyalectans turnover, in some cases stable proteolytic fragments are generated. Members of the MMP and ADAMTS families have been demonstrated to cleave hyalectans in vitro and are thus believed to be responsible for their degradation in vivo. Interestingly, proteolytic fragments generated after in vitro cleavage correlate to those detected in protein extracts from different tissues underlining the physiological relevance of this proteolytic process.

In embryonic and early postnatal brain, neurocan is mainly present in its uncleaved 250 kDa form, while in the adult brain the two cleavage products of 130 and 150 kDa predominate (Matsui et al., 1994). The physiological significance of this developmentally regulated proteolysis is unclear at present; similarly the enzymes involved in neurocan cleavage have not yet been identified.
Loss of aggrecan due to proteolytic cleavage has been described in arthritic cartilages. In particular, cleavage within the interglobular domain (IGD) located between the G1 and G2 domain results in the release of the glycosaminoglycan rich fragment, which is important for the mechanical properties of the cartilages. Members of the MMP and ADAMTS (ADAMTS-1, ADAMTS-4 and ADAMTS-5) families cleave within the IGD at two distinctive sites, as well as at other sites of the aggrecan core protein. Thus deregulation of ADAMTS and MMP activities are thought to be the major cause of human arthritis.

*In vitro* susceptibility to digestion by MMPs and ADAMTS has also been demonstrated for brevican. In the adult rat brain, brevican is detectable as full-length protein of 145 kDa and as two proteolytically cleaved forms of 90 and 50 kDa probably generated by ADAMTS-4 activity (Nakamura et al., 2000). Interestingly, brevican cleavage has been correlated with an invasive phenotype of gliomas (Matthews et al., 2000).

![Figure 12: The sites of ADAMTS cleavage (blue arrowheads) that have been characterized in the different hyalectans.](image)

### 1.3.2.2. Versican Degradation

The enzymatic pathways involved in the degradation of aggrecan have been described in great detail (Lemons et al., 2001; Sandy and Verscharen, 2001). In contrast very little is known about the versican turnover. Several versican degradation products have been found in different tissues, however the metabolic origin of these proteins and their biological relevance are still rather vague. The recent finding that members of the ADAMTS family are able to cleave other hyalectans at specific sites suggests that also versican may be degraded in a similar manner. Based on sequence alignments of the known ADAMTS cleavage sites found in aggrecan and brevican a rather loose consensus sequence for cleavage: pt(V/I)XX(V/I)(t/d)XXlE*Xrg has been proposed (asterisk represent the scissile bond, uppercase residues are 100 % conserved, lowercase residues are 50 % or more conserved and X represent nonconserved residues) (Sandy et al., 2001). Interestingly two potential cleavage sites were detected in the GAG-α and GAG-β domains of versican, respectively. *In vitro* experiments demonstrated that those sites are indeed recognized by different ADAMTSs. To date specific cleavage of recombinant versican fragments by ADAMTS-1 (Sandy et al., 2001), ADAMTS-4 (Westling...
et al., 2004), and ADAMTS-9 (Somerville et al., 2003) have been demonstrated. With antibodies raised against the potential neo-epitopes generated by ADAMTS proteolysis of versicans the specific catabolic products have been identified in various tissues like human aorta (Sandy et al., 2001), human brain (Westling et al., 2004) and fetal human skin (Sorrell et al., 1999). In particular a 70 kDa and a 220 kDa fragment derived from versican V1 and versican V0 cleavage, respectively, were extracted from human aorta, whereas GHAP, a 64 kDa glial hyaluronan-binding glycoprotein isolated from brain tissues appears to be an ADAMTS cleavage product of versican V0 and/or V2. Interestingly, at least ADAMTS-4 is present in all of these tissues. It seems therefore likely that ADAMTS-4 and eventually also other members of the “aggrecanase” group (ADAMTS-1, -5, and -9) may indeed be responsible for the turnover of versicans, in vivo. Additional, not ADAMTS-cleavage-related fragments that are usually also present in tissue extracts suggest an involvement of other proteinases such as MMPs (Bignami et al., 1994; Perides et al., 1995; Halpert et al., 1996; Passi et al., 1999) in the complete degradation of versican.

ADAMTS mediated cleavage may be the initial step of the versican breakdown similar to the process leading to aggrecan degradation in arthritis (Caterson et al., 2000). This cleavage separates the N- and C-terminal domains that possess specific matrix binding properties. Hence, it may be sufficient to change the functions of versican in linking other extracellular matrix molecules and may affect interstitial tissue structure and cell anchorage. It was demonstrated for example that versican cleavage, probably mediated by ADAMTS-1, is a crucial event during cumulus oocyte complex (COC) expansion and ovulation (Russell et al., 2003).

Whether ADAMTSs mediated proteolysis also plays a role in the regulation of versican activities in biological processes such as axonal out growth and neural crest cell migration is currently not known.

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**Figure 13:** Versican V2 fragments identified in adult brain extracts (Westling et al., 2004).
2. Gene Targeting Methods in Mice

Mutation analysis, defined as the inactivation or modification of a gene and subsequent study of the consequences of the mutation in the living organism, is a powerful tool to study the in vivo function of genes. In recent years homologous recombination in mouse embryonic stem cells has become a routine technique to generate mutated animals. Several strategies have been developed to inactivate genes in a constitutive, tissue and/or time specific manner. This has made the mouse a highly valuable study animal to explore gene functions and to generate models for human diseases.


In the classical approach targeted gene inactivation is accomplished by homologous recombination in ES cells followed by generation of knockout mice. Gene disruption in these null mutants is generally achieved by 1) replacing a crucial gene segment with a mutant version of this segment or 2) by deleting a suitable fragment of the gene of interest. In many experiments a selectable gene encoding for a resistance marker is inserted into the targeted locus in a manner that allows its expression (and hence cellular selection) while inactivating the targeted gene. However this strategy could be problematic mainly because of the strong promoter used for expression of the selection marker locus that may interfere with the expression of genes adjacent to the targeted one (Lerner et al., 1993; Rajewsky et al., 1996). Also other mechanisms leading to leaky expression or translation of modified proteins may interfere with the interpretation of the knockout phenotype. Such events are difficult to predict and may include alternative splicing of the targeted exons, use of alternative promoters or ATG start signals and generation of truncated proteins that partially retain the original activity (Kwan, 2002). In some cases such problems can be overcome by the complete deletion of the gene. However this approach is technically almost impossible for large genes, furthermore such deletions may influence the expression of unrelated genes that have regulatory elements within the deleted fragment (Kwan, 2002). Although the simple inactivation of a gene is a well-established technique, it has several limitations. In some cases gene disruption may result in embryonic or neonatal lethality, compromising analysis at later stages. Alternatively the loss of gene product may be compensated by other genes belonging to the same family or parallel pathways (Lobe and Nagy, 1998). Furthermore the gene of interest may have a wide expression pattern and its general inactivation may produce a complex phenotype involving different tissues. Finally to understand the function of a gene in a particular process it may be useful to inactivate gene function only at a particular time point or at a particular developmental stage (Bockamp et al., 2002). For all these reason several techniques have been developed to generate mouse models allowing gene inactivation in selected tissues and/or at a specific time point.

2.2. Conditional Gene Targeting Strategies

2.2.1. The Cre/lox P Flp/FRT Systems

The first system used to generate conditional gene inactivation in the mouse was the Cre-lox P recombination system (Lakso et al., 1992). This system relies on Cre, a 38 kDa recombinase protein encoded by the E.Coli bacteriophage P1, which recognizes and mediates
site-specific recombination between 34 bp sequences called lox P (locus of crossover (x) in P1). The lox P sequence consists of a core spacer of 8 bp flanked by palindromic sequences of 13 bp. The 8 bp non-palindromic portion defines the orientation of the lox P sites (Kuhn and Torres, 2002).

Depending on the orientation of the lox P sites Cre recombinase mediate different events on DNA targeting sequences (Figure 14). When a linear DNA fragment is flanked by lox P sites with the same orientation, Cre mediates an intramolecular recombination between the lox P sites. This results in the excision of the “floxed” DNA fragment as a circular molecule carrying one lox P site while the other lox P site remains in the original DNA sequence. With lower efficiency, Cre mediates the reverse reaction by inserting a circular DNA molecule into a linear DNA molecule each possessing one lox P site. Alternatively Cre recombinase mediates intermolecular recombination between two linear DNA molecules each containing a lox P site. Finally if two lox P sites are localized on the same DNA molecule but in the opposite orientation, Cre mediated recombination leads to an inversion of the floxed DNA fragment.

Figure 14: The Cre/lox P Flp/FRT systems. (A) Structure of the lox P and FRT sites. (B) Cre mediated recombination events (Kwan, 2002).

Since the demonstration that Cre mediated site specific recombination works efficiently in mammals, it has rapidly become the most exploited system to achieve spatio-temporal deletion of genes in mouse.

An alternative system based on the yeast FLP/frt recombinase system has also been used to achieve conditional gene inactivation. Similar to the Cre recombinase, the S.cerevisiae recombinase Flp causes recombination between "Flp recombination target" (FRT) sites. Flp mediated recombination in vivo was initially quite inefficient due to thermal instability of the wild type enzyme at 37°C (Buchholz et al., 1996). Recently a thermostable variant of Flp has been generated, termed Flpc. This modified enzyme shows in vivo recombination efficiencies similar to that of Cre (Rodriguez et al., 2000).
2.2.2. Tissue Specific Gene Inactivation

Strategies to achieve tissue specific gene inactivation start with the generation of a mouse in which two lox P sites are introduced that flank a functionally essential part of the gene of interest. Proper insertion of the 34 bp long lox P sites leads to only minimal changes in the genome while preserving the function of the targeted gene. Consequently, the loxP carrying strain should be indistinguishable from wild type mice. Tissue specific gene inactivation is subsequently achieved by crossing the “floxed” mouse with a transgenic strain that expresses Cre under the control of a tissue specific promoter. This will result in double mutant mice in which deletion of the floxed gene or a floxed gene fragment happens only in those tissues that express the Cre recombinase. Finally the mice should be crossed to obtain mice homozygous or alternatively hemizygous (having a floxed and a deleted allele) for the floxed locus and at least hemizygous for the Cre transgene. To date several mouse lines have been generated and used in conditional knockout experiments, some of them are listed in the following databases. A. Nagy, private database: http://www.mshri.on.ca/nagy/cre.htm; European mouse mutant archive (EMMA): http://www.emma.rm.cnr.it/Home.html; the Jackson Laboratory, Bar Harbor: http://www.jax.org.

![Diagram of gene inactivation strategies](image)

**Figure 15:** Strategies for tissue specific gene inactivation. (A) 2-loxP strategy. The selection marker is still in the final targeted conditional null allele. (B) 3-loxP strategy. After gene targeting in vitro transient Cre expression will generate three type of ES cell subclones due to partial recombination: one harboring the desired allele (type II), the other two are null allele (type I, and type III) (C) 2-loxP-2-FRT strategy. A two step strategy using both the Cre-loxP and Flp-FRT recombination system. The flanked selection cassette is removed by Flp-mediated deletion after either in vivo or in vitro after the gene replacement step. Red arrows: loxP sites; dark blue circles: FRT sites.

2.2.3. Regulated Gene Inactivation

2.2.3.1. Transcriptional Regulation of Cre Activity

Several strategies combining tissue specific promoters with exogenous induction of Cre activity have been pursued to enhance the potential of site specific recombination in knockout experiments. Several systems to control Cre activity have been tested, these include
regulation of Cre activity by ligand dependent transcriptional activation or by posttranslational modulation of Cre by synthetic ligands.

In the first inducible Cre mouse, the interferon-responsive promoter of the Mx1 gene was used to drive Cre expression (Kuhn et al., 1995). However excision of floxed alleles upon application of interferon was limited to the liver and immune system. Another improved system based on the tetracycline-mediated regulation of Cre expression has been developed and successfully applied to drive inducible spatio-temporal gene inactivation. Two variants of the tetracycline mediated regulation of gene expression, the tet-off and tet-on systems, have been put into practice. Both systems rely on two central components: (1) a tetracycline responsive transcriptional activator that is a hybrid protein composed of the E. Coli tetracycline repressor fused to the VP16 transactivator domain of the herpes simplex virus, and (2) a transactivator dependent promoter consisting of a minimal promoter fused to tet operator (tet O) sequences.

In the original tet-off system (Gossen and Bujard, 1992) tetracycline or the tetracycline analogue doxycycline (DOX) prevent transcription by binding to the transactivator, tTA. Instead expression of the responder gene is induced upon ligand removal. A more recent variant of this system, the tet-on system (Gossen et al., 1995), employs a reverse tTA (rtTA), which become active only in presence of tetracycline or its derivatives. Thereby in the tet-on system expression of the responder gene is normally silent but can be induced by ligand administration.

The tet systems can be used to obtain spatio-temporally controlled gene inactivation. For this purpose triple transgenic animals should be generated, which carry (1) a tTA or rtTA gene driven by a tissue specific promoter, (2) a Cre gene under the control of the tet O promoter and (3) a lox P flanked target sequence (Branda and Dymecki, 2004).

In several tet O::Cre mouse strains inefficient regulation of Cre expression demonstrated the importance of the integration site of the tet O controlled transcription unit. In these cases the presence of neighboring endogenous enhancers directly activated the tet O promoter, thus disrupting tight regulation of Cre expression (Leneuve et al., 2003).

Interestingly, a new tet O::Cre mouse line has recently been described, termed LC-1, in which Cre expression is tightly controlled by the tet system (Schonig et al., 2002). Probably in the future, insertion of tet O::Cre expression cassettes into the LC-1 locus, coupled with tissue restricted tTA or rtTA expression will have broad use in spatio-temporal inactivation of floxed genes.

2.2.3.2. Post-Translational Regulation of Cre Activity

Besides controlling Cre or Flp gene expression using the tet system or using tissue specific or inducible promoters, modified recombinases have been developed to modulate their activity by exogenous effector molecules.

Several Cre chimaeric proteins were produced by fusing the Cre recombinase to the ligand binding domain (LBD) of different steroid receptors. In absence of the steroid ligand heat shock proteins (HSPs) bind to the Cre fusion protein masking its enzymatic activity. Administration of the ligand results in the dissociation of HSPs from the Cre fusion protein, which is consequently translocated to the nucleus where it mediates site-specific recombination. To avoid activation by endogenous ligands, mutant LBDs responsive to synthetic ligands were used.

Three different variants of a tamoxifen inducible estrogen receptor (ER) mutant were developed to achieve inducible Cre recombination in many tissues of adult animals. These include (1) a mouse ERTM with a G525R mutation (Danielian et al., 1998), (2) a human ERT
with a G521R mutation (Logie and Stewart, 1995; Metzger et al., 1995), and a human ERT2 containing three mutation G400V/M543A/L544A that in comparison to the ERT variant shows a approximately 10-fold improved sensitivity for both nuclear translocation and recombinase activity (Feil et al., 1997; Indra et al., 1999).

Alternatively, a modified human progesterone-binding domain of the progesterone receptor (PR) was used. Also in this case mutations were introduced allowing induction by the synthetic glucocorticoid receptor antagonist RU486 but not by endogenous progesterone or glucocorticoids. The first mutated variant, termed Cre-PR1 (Kellendonk et al., 1996) displayed inducible Cre activity in tissues like adult epidermis but showed a considerable degree of leakiness. Lately, an improved CRE-PR version, called CRE*PR has been developed that shows a reduced background activity and improved RU486 sensitivity (Wunderlich et al., 2001).

The third in vivo inducible Cre fusion protein system uses a mutant LBD of the human glucocorticoid receptor (GRdex) (Brocard et al., 1998). In this case the Cre fusion protein is activated by the synthetic ligands dexametasone, triamciclone acetonide or RU38486, but is insensitive to the natural ligands corticosterone, cortisol or aldosterone.

![Figure 16: Strategies for the spatio-temporal control of Cre mediated gene inactivation.](image)

The gene targeting technology has been successfully applied to study gene function in the nervous system and to develop animal models for neurological diseases. In particular the development of conditional and regulated strategies has provided temporal and spatial control of gene inactivation. This is specially relevant to study the function of genes in the nervous tissue where the discrete functional activities of the diverse groups of brain cells requires highly specific cellular targeting. However many tissue specific systems developed so far still show a poor spatial resolution of gene inactivation, whereas most of the inducible systems have disadvantages linked to background activity in the absence of the inducer, limited efficiency of recombination, and toxicity of the inducer. Thus further development of the conditional gene targeting technique is still required in order to improve cellular restriction and temporal regulation of mutations.
PART I: Generation of a Floxed Allele of Versican for Cre-Mediated Conditional Knockout in the Mouse

1. Introduction

Interactions between neural cells and their surrounding environment play crucial roles during morphogenesis as well as for the maintenance of functioning tissue structures in the adult organism. The signals influencing the individual cellular behaviour may either be received over a short-range through cell-cell-and cell-matrix-contacts or may be transmitted over longer distances by means of diffusible factors that form gradients. In the developing nervous system, various components of the extracellular matrix act on short-range by modulating cell adhesion, migration and axonal guidance, while they may primarily function in restricting axonal growth and controlling synaptic plasticity in the adult CNS. The extracellular matrices of the brain and spinal cord have rather unique molecular compositions that greatly differ from the connective tissues of other organs. The main ECM-components in the central nervous system, are hyaluronan (hyaluronic acid), proteoglycans, link proteins and tenascins, while matrix proteins common in other tissues, such as fibrillar collagens or fibronectins are nearly absent (Novak and Kaye, 2000).

Among the great variety of proteoglycans present in the developing and mature central nervous tissue, the family of hyalectans (or lecticans) (Ruoslhti, 1996; Iozzo, 1998) have gained particular attention in regard to their putative role in axonal guidance during development (Margolis and Margolis, 1993; Margolis and Margolis, 1997; Bandtlow and Zimmermann, 2000) and in structural stabilization of the mature nervous system (Hockfield et al., 1990; Yamada et al., 1997). Four family members are currently known, namely aggrecan (Doege et al., 1991), brevican (Yamada et al., 1994), neurocan (Rauch et al., 1992) and versican (Zimmermann and Ruoslhti, 1989). Hyalectans are characterized by the presence of highly conserved N- and C-termial domains that mediate interaction with hyaluronan and tenascins (Iozzo, 1998; Bandtlow and Zimmermann, 2000), respectively. In contrast, the central glycosaminoglycan attachment regions of the hyalectans are highly diverse in terms of size and sequence.

The glycosaminoglycan middle region of the versican core protein consists of two extended subdomains, designated GAG-α and GAG-β that are encoded by two large exons. In mammals, alternative splicing of these two exons gives rise to four versican isoforms (Dours-Zimmermann and Zimmermann, 1994) (Zako et al., 1995). The largest one (V0) includes both the GAG-α and GAG-β domains and carries 17 to 23 chondroitin sulfate chains. The shorter V1 and V2 isoforms, contain either the GAG-β or GAG-α domain, respectively. Thus the number of chondroitin sulfate chains is reduced to 12 to 15 in V1 and 5 to 8 in V2. The fourth versican isoform (V3) is composed of only the N- and C-terminal globular domains and is devoid of glycosaminoglycan chains.

All hyalectans are expressed in the developing and/or mature brain with specific expression profiles (Rauch et al., 1991; Li et al., 1996; Yamada et al., 1997; Schmalfeldt et al., 1998). In the rat brain, neurocan and versican V0/V1 are expressed almost exclusively during embryonic and early postnatal development. They seem to be replaced in the adult brain by versican V2, brevican and aggrecan (Milev et al., 1998). Expression of versican V2 is restricted to the mature central nervous system. It is expressed by oligodendrocytes and consequently present in the myelinated fiber tracts where it accumulates at the node of Ranvier (Oohashi et al., 2002). It seems thus conceivable that versican V2 may play a significant role in reducing the axon outgrowth activity and...
maintaining a relative stable neuronal circuitry in the mature CNS. In contrast the other versican isoforms have a much wider tissue distribution (Yamagata et al., 1989; Bode-Lesniewska et al., 1996). In adults, versican V0/V1 is mostly associated with blood vessels, elastic connective tissues and the basal layer of the epidermis. In the adult nervous system these large isoforms are mainly found in the meninges and around cerebral blood vessels likely being expressed by astrocytes. During development of the PNS versican V0/V1 is present in tissues that act as non-permissive barriers to migrating neural crest cells and extending axons of motor and sensory neurons (Landolt et al., 1995), suggesting an inhibitory function for these two isoforms. Recently, the hypothesis that versicans may directly participate in the control of cell and axon migration has obtained further support by data from in vitro studies. In experiments using stripe choice assays we could demonstrated that isolated versican V2, versican V1 and a mixture of versican V0/V1, inhibited the migration of neural crest stem cells and interfered with the outgrowth of neurites from retinal and dorsal root ganglion explants (Schmalfeldt et al., 2000) (S. Dutt-Raghav, manuscript in preparation). Similarly, versican practically reduced the substrate-adhesion of cells of various origins (Yamagata et al., 1989) (S. Dutt-Raghav, manuscript in preparation).

Despite of this increasing evidence that versican may act as non-permissive substrate of cell and axon adhesion, the confirmation in vivo is still missing. Unfortunately, constitutive inactivation of the versican gene in mice leads to early embryonic lethality due to a heart malformation (Mjaatvedt et al., 1998). We therefore initiated a conditional knockout approach that should allow to overcome the lethal phenotype and permit the study of the functions of versicans during development and maturation in vivo. For this purpose we have generated a recombinant mouse strain that carries two loxP sites flanking the versican promoter. By crossing this strain with transgenic mice expressing the Cre recombinase under tissue-specific promoter, we will try now to limit the versican gene inactivation to the nervous system.
2. Materials and Methods

2.1. Molecular Biology and Cell Culture

2.1.1. Cloning and Sequence Analysis of the Targeting Region

The 10 kb long region downstream of the versican promoter was obtained by long-distance PCR (LD-PCR) using primers specific for the first (Exon I/II up) and second (Exon I/II low) versican Exon, respectively.

In order to obtain an 11 kb sequence fragment upstream of the versican promoter, a strategy involving several rounds of inverse PCR (Inv. PCR) was applied. For this purpose about 1 µg of genomic DNA previously digested with an appropriate restriction enzyme, was incubated o/n at 14°C with 25 U T4 ligase (Roche Diagnostics) in 400 µl 1x ligation buffer. After phenol/chloroform extraction and ethanol precipitation, 50-100 ng of circularized DNA were used as template for inverse PCR. Table 2.1 lists the sequence of the different primer pairs and summarizes the reaction conditions. Further details about primer position and restriction enzymes are described in the result section.

Table 2.1: Primers pairs and reaction condition used to subclone the targeting region

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>PCR Type</th>
<th>Product length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon I/II up:</td>
<td>LD-PCR</td>
<td>10.7 kb</td>
<td>35</td>
</tr>
<tr>
<td>Exon I/II low:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inv. PCR 1 up:</td>
<td>Inv. PCR</td>
<td>3.2 kb</td>
<td>35</td>
</tr>
<tr>
<td>Inv. PCR 1 low:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inv. PCR 2 up:</td>
<td>Inv. PCR</td>
<td>2.2 - 6.5 kb*</td>
<td>35</td>
</tr>
<tr>
<td>Inv. PCR 2 low:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(LD-PCR): Long distance PCR using the TaKaRa LA enzyme (1x PCR buffer, 2.5 mM MgCl₂, 0.2 µM dNTPs, 0.2 µM primers, 100-200 ng template, 2.5 U Polymerase in a total volume of 50 µl). Cycling conditions: 95°C 1 min, 68°C 3 min 30 s. Initial denaturation 94°C 2 min; final extension 68°C 7 min. After 15 cycles 20 sec step extension per cycle.

(Inv.PCR): Inverse PCR using the TaKaRa LA enzyme (1x PCR buffer, 2.5 mM MgCl₂, 0.2 µM dNTPs, 0.2 µM primer, 100-200 ng template, 2.5 U Polymerase in a total volume of 50 µl). Cycling conditions: 95°C 1 min, 68°C 3 min 30 s. Initial denaturation 94°C 2 min; final extension 68°C 7 min. After 15 cycles 20 sec step extension per cycle.

The different PCR-amplified fragments were T/A subcloned into pGemTeasy vector (Promega). Sequencing was done on an ABI 377 DNA Sequencer using dye terminator chemistry (Perkin Elmer/ABI, Foster City, CA).

2.1.2. Preparation of the Targeting Constructs

Isogenic targeting vectors for conditional gene ablation were prepared for mouse 129/Sv and C57BL/6 genetic backgrounds. The targeting constructs were assembled from four different genomic fragments corresponding to: a) the 5' arm, b) the promoter/exon I fragment, c) the first and d) the second half of the 3' arm (Figure 2.1). These fragments were amplified from genomic DNA extracted from R1 (129/Sv) or Bl6-III (C57BL/6) ES cells and subcloned into the pGemTeasy vector (Promega). These genomic clones, forming the basis to
construct the targeting vectors, were named p5'Arm, pPro, p1/2-3'Arm and p2/2-3'Arm. Table 2.2 summarizes primers and reaction conditions used to synthesize the four genomic DNA fragments.

Table 2.2: Primers and reaction conditions used to generate the targeting vectors

<table>
<thead>
<tr>
<th>Primers Pairs*</th>
<th>PCR Type</th>
<th>Product length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' Arm BstZI up</td>
<td>LD-PCR</td>
<td>4.2 kb</td>
<td>20</td>
</tr>
<tr>
<td>5' Arm BstZI low</td>
<td>LD-PCR</td>
<td>0.75 kb</td>
<td>20</td>
</tr>
<tr>
<td>Promoter Sall up</td>
<td>LD-PCR</td>
<td>3.2 kb</td>
<td>20</td>
</tr>
<tr>
<td>3' Arm EcoRV up</td>
<td>LD-PCR</td>
<td>2.8 kb</td>
<td>20</td>
</tr>
<tr>
<td>SacI low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(*): Bases complementary to versican gene sequences are written in capital letters. Red are restriction site sequences.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(LD-PCR): Long distance PCR with TaKaRa LA enzyme (1x PCR buffer, 2.5 mM MgCl2, 0.2 μM dNTPs, 0.2 μM primers, 100-200 ng template, 2.5 U Polymerase in a final volume of 50 μl). Cycling conditions: 95°C 1 min, 68°C 5 min 30 s. Initial denaturation 94°C 2 min; final extension 68°C 7 min. After 15 cycles 20 sec step extension per cycle.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 2.1: PCR cloning of the different genomic fragments. P: promoter; I: exon1; B: BamHI; Bs: BstZI; E: EcoRI; N: NotI; S: Sall; Sa: SacI; Sp: SpeI; X: Xhol.](image)

In the next step a vector (plox) containing a loxP site flanked by the restriction sites Xhol and SpeI, was generated by inserting a hybridized cassette (sense oligo: 5' TCG ACA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT CGA T 3'; antisense oligo: 5' ATC GAT AAC TTC GTATAA TGT ATG CTA TAC GAA GTT ATG 3') between the Xhol and the EcoRV sites of the pBluescript vector (Invitrogen). The Sall-Xhol fragment of the pPro construct, corresponding to the versican promoter and part of the 5' untranslated region, was ligated into this new vector (Figure 2.2). This way the loxP site was inserted within the first exon of the versican gene, resulting in the pPro/lox construct.

The complete 3'-arm of the targeting construct was obtained by adding the SacI-SacI fragment of the p2/2-3'Arm to the p1/2-3'Arm vector. The full-length 3'-arm was isolated
from this new plasmid (p3’Arm) by EcoRV SpeI digestion and inserted into the corresponding
sites of the pPro/lox vector giving rise to the pProm-lox-3’Arm construct.
The NotI-BstZI fragment of the p5’Arm was initially inserted in the NotI site of a pBluescript
vector carrying a neo-tk cassette floxed by lox P sites, giving rise to the p5’Arm-neotk
construct. Both the neo- and the tk-gene are under the control of the strong Herpes Simplex-
thymidine kinase promoter.
In the last step the SalI-SalI fragment of the pProm-lox-3’Arm vector was inserted in the
p5’Arm-neotk to generate the final targeting construct for conditional gene inactivation of the
versican gene.

Figure 2.2: Assembly of the targeting vector. Green box: neo-tk selection cassette under the control of the HSV-
tk promoter; gray box: pGemTeasy sequence; black box: pBluescript sequence; red arrow: loxP sites; B: BamHI;
Bs: BstZI; E: EcoRI; N: NotI; S: SalI; Sa: Sacl; Sp: SpeI; X: Xhol.
2.1.3. Cell Culture and Electroporation

2.1.3.1. Embryonic Stem Cells Lines

The B1/6-III cell line was generated by B. Ledermann and K. Bürki and is derived from the C57BL/6 mouse strain (Ledermann and Burki, 1991) (kindly provided by B. Ledermann). B1/6-III ES cells were cultivated on feeder cells in KNOCKOUT D-MEM medium (Gibco, Life Technologies) supplemented with 10% Knockout serum replacement (KNOCKOUT SR, Gibco, Life Technologies), 2 mM Glutamax (Gibco, Life Technologies), 0.1 mM β-mercaptoethanol and 1000 U/ml Leukemia inhibitory factor (Lif, kindly provided by B. Ledermann).

The R1 ES cell line was derived from a 129/Sv mouse strain by A. Nagy (Nagy et al., 1993) (kindly provided by C. Brakebusch). R1 cells were grown on feeder cells in D-MEM medium supplemented with 20% Fetal Calf Serum (FCS, Winiger AG, Bio-Consulting), 2 mM Glutamax (Gibco, Life Technologies), 1x non-essential amino acids (Gibco, Life Technologies), 0.1 mM β-mercaptoethanol and 1000 U/ml Lif.

2.1.3.2. Preparation of Feeder Cells

Mouse embryonic fibroblasts (MEF) were derived from day 13.5-14.5 fetuses of Perforin K.O. mice (Kagi et al., 1994) according to the protocol of R. Fässler (Talts et al., 1999). Prior to co-culturing with ES cells, MEF were mitotically inactivated by γ-irradiation with 1500 rad. MEFs were plated at least 1 day before seeding of the ES cells and used as feeder cells up to 2 weeks.

2.1.3.3. Electroporation of Embryonic Stem Cells

For homologous recombination 5 x 10⁶ (B1/6-III ESC) or 2 x 10⁷ (R1 ESC) exponentially growing ES cells were transfected with 20 µg or 60 µg of linearized targeting construct, respectively. Electroporation was performed using a Bio-Rad Gene Pulser II device applying the following conditions: 0.24 kV, 500 µF (B1/6-III ESC) or 0.8 kV 3 µF (R1 ESC). Aliquots of electroporated cells were grown on 10 cm dishes for 6-8 days in growth medium supplemented with 500 µg/ml Geneticin (Gibco, Life Technologies). Colonies surviving the selection regime were transferred under a stereomicroscope into 24-well plates and expanded for freezing and genomic DNA preparation.

For the transient Cre expression 2 x 10⁶ cells were electroporated with 5-10 µg of the pMC-Cre (Gu et al., 1993) vector. 48h after electroporation cells were seeded at 1:50-1:100 dilution and subjected to negative selection with 2 µM Gancyclovir (Cymevene®, Syntex Pharm AG) for 3 days. After 3-5 additional days aliquots of the clones were frozen and the rest processed for DNA preparation.
2.1.4. Analysis of Embryonic Stem Cell Clones

2.1.4.1. Preparation of Genomic DNA

To prepare genomic DNA for PCR screening, single ESC colonies surviving antibiotic selection were transferred into 24-well plates. 2-4 days after picking cell clones, half of the cells in the well were frozen and half was further expanded until they reached confluence. Genomic DNA was prepared directly in the 24-well plates by incubation at 37°C overnight in PCR-lysis buffer (100 mM Tris-HCl pH 8.5, 0.2% SDS, 200 mM NaCl, 100 μg/ml Proteinase K). Following isopropanol precipitation, DNA was resuspended in TE buffer. For Southern blot analysis, PCR positive colonies were expanded in 10 cm dishes to confluence. Cells were collected by trypsinization and lysed by Proteinase K digestion (100 μg/ml) at 55°C o/n in extraction buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, pH 8). Genomic DNA was finally purified by phenol/chloroform extraction followed by ethanol precipitation.

2.1.4.2. PCR Screening

Homologous recombined clones were identified by LD-PCR screening using primers specific for the sequence upstream of the targeted region and within the proximal part of the neo-tk cassette (primer pair: 5' up / 5' low). Correct integration was independently confirmed using primers specific for the sequence within the distal part of the neo-tk cassette and for downstream of the 3'-arm (primer pair: 3' up / 3' low).

Floxed clones generated by transient Cre expression were identified with primers specific for the sequence upstream and downstream of the new loxP site generated by removal of the selection cassette (primer pairs: P1/2 up / P1/2 low). The presence of the second loxP site was confirmed using primers annealing up- and downstream of the corresponding site. Furthermore all positive clones were verified by direct sequencing of the PCR products.

All PCR conditions and primers are listed in Table 2.3, detailed information about primer positions are given in the results section.

Table 2.3: PCR primers and cycling conditions used for ES cells screening

<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>PCR Type</th>
<th>Product length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' up:</td>
<td>GGGACACACTAATGGGGTCTTTTTGA</td>
<td>LD-PCR</td>
<td>4.8 kb</td>
</tr>
<tr>
<td>5' low:</td>
<td>GAGATGGGGAGGCTAAACTGAAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3' up:</td>
<td>TGCCAGTGTAGCGGGAATA</td>
<td>LD-PCR</td>
<td>7.2 kb</td>
</tr>
<tr>
<td>3' low:</td>
<td>CACCCCTGCAAAAAATCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1/2 up:</td>
<td>TTCAGTTGCGGAGGAGCATT</td>
<td>PCR</td>
<td>159 bp* / 295 bp#</td>
</tr>
<tr>
<td>P1/2 low:</td>
<td>GGCAGCAACAGCACAGGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P3 up:</td>
<td>CGCGCAGCTGTGAGCA</td>
<td>PCR</td>
<td>201 bp* / 245 bp#</td>
</tr>
<tr>
<td>P3 low:</td>
<td>GCAATTTTTCGGGGGTGAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(LD-PCR): Long distance PCR with TaKaRa enzyme (1x PCR buffer, 2.5 mM MgCl₂, 0.2 μM dNTPs, 0.2 μM primers, 100-200 ng template, 2.5 U Polymerase in a total volume of 50 μl). Cycling conditions: 95°C 1 min, 68°C 3 min 30 s. Initial denaturation 94°C 2 min; final extension 68°C 7 min. After 15 cycles 20 sec step extension per cycle.

(PCR): Standard PCR with Taq GOLD Polymerase (1x PCR buffer, 2.5 mM MgCl₂, 2.5 mM dNTPs, 0.2 μM primers, 50-100 ng template, 1 U Polymerase in a total volume of 20 μl). Cycling conditions: 95°C 1 min, 65°C 1 min. Initial denaturation 94°C 10 min; final extension 72°C 7 min

*#/ Fragment length of the wild type and targeted allele respectively
2.1.4.3. Southern Blotting

For Southern blot analysis 5 μg of genomic DNA digested overnight with the corresponding restriction enzyme, were separated on 0.5% agarose gel. Following vacuum-transfer to a nylon membrane (Nylon membrane positively charged, Roche Diagnostic), DNA was hybridized overnight at 68°C with the appropriate Dig-labeled probe in hybridization buffer (5 x SSC, 0.5 % blocking reagent, 0.1 % N-lauroylsarcosine, 0.02 % SDS). After extensive washing, DNA was visualized with alkaline phosphatase conjugated anti-Dig antibody (Roche Applied Science) and a chromogenic detection method using a mixture of nitro-blue tetrazolium (NBT, Roche Applied Science) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Roche Applied Science) as substrate.

The correct structure of the recombined allele was verified by hybridization with two different probes located up- and downstream of the target position. An additional internal probe was used to identify clones with single genome integration of the targeting construct.

Correct deletion type arising from transient Cre expression was confirmed with a probe (Probe A) hybridizing within the first intron of the versican gene.

All PCR conditions and primers used to generate the different DIG-labeled probes are listed in Table 2.4, while the correct position of the probes is shown in the result section (Figures 3.4 and 3.5).

Table 2.4: Primer and reaction conditions used to generate DIG labeled DNA probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer Pairs</th>
<th>PCR Type</th>
<th>Product length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>BamHI up: TGGGTTACCTGGGTAATAGAAAT</td>
<td>Dig-PCR</td>
<td>0.65 kb</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>BamHI low: GAGGTTATACTGGAAGCTGCTGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoRV</td>
<td>EcoRV up: AGTAAATTAGTAGCCCAATCCAG</td>
<td>Dig-PCR</td>
<td>0.7 kb</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>EcoRV low: GAGAGACACTAGTTGCCAAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-Probe</td>
<td>NT Probe up: GGAGCCTGGGGTGGTGAC</td>
<td>Dig-PCR</td>
<td>1 kb</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>NT Probe low: GCTGGGGGCTTTCCAGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probe-A</td>
<td>Probe-A up: ACCCCCTAGGCAATG</td>
<td>Dig-PCR</td>
<td>0.8 kb</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Probe-A low: GTCCACGCCCATGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Dig-PCR): DIG labeling PCR using the Taq GOLD Polymerase (1x PCR buffer, 2.5 mM MgCl₂, 0.2 μM dATP, 0.2 μM dGTP, 0.2 μM dCTP, 0.15 μM dTTP, 0.05 μM DIG-dUTP, 0.2 μM primers, 10 ng template, 5 U Polymerase in a total volume of 100 μl). Cycling conditions: 95°C 1 min, 68°C 4 min. Initial denaturation 94°C 10 min; final extension 72°C 7 min.

2.1.4.4. Karyotype Analysis

Synchronized ES cell cultures were treated with 5 μg/ml demecolcine solution (Sigma) and incubated 3 h at 37°C. Collected cells were resuspended in 0.56% KCl and incubated at 37°C for 10 min to allow cell swelling. Pelleted cells were then fixed 3 times in icecold methanol/acetic acid (3:1) for 10 min. Fixed cells were dropped from a height of about 50 cm onto Superfrost slides (Merck) to spread the chromosomes. After drying, the chromosome spreads were stained with 2 % Giemsa stain solution (Sigma) for 30 min. After two washes with tap water, slides were air dried and mounted with water soluble mounting medium.
2.1.5. Generation of Chimera, Breeding and Identification of Targeted Mice

Injections of ES clones cells into FVB (BL6/III) or C57BL/6 (R1 ESC) strain blastocyst were done at the Institute of Laboratory Animal Science (University of Zurich), or at the Department of Molecular Medicine (Max Planck Institute for Biochemistry, Martinsried, Germany), respectively. Chimeras derived from Bl/6-III or R1 ES cells were crossed with wild type C57BL/6 or 129/Sv mice. Offsprings resulting from germline transmission were identified on the basis of their fur color (C57BL/6) or by genotyping (129/Sv). For the identification of targeted mice, genomic DNA was extracted from tail biopsies. Template material was incubated at 55°C overnight in PCR-lysis buffer (100 mM Tris-HCl pH 8.5, 0.2 % SDS, 200 mM NaCl, 100 µg/ml proteinase K). After inactivation of the proteinase K by 10’ incubation at 95°C, aliquots of the DNA solution were analyzed by PCR using the same primer pairs used for the screening of the floxed clones (P1/2 up / P1/2 low and P3 up / P3 low).

2.2. Generation of Antibodies Against Mouse Versicans

The rabbit anti mouse versican GAG-α antibody was previously prepared in our lab (Schmalfeldt et al., 2000). It is has been raised against a bacterial fusion protein including the amino acid residues 362 to 585 of the mouse versican (Genbank Q62059). The bacterial expression vector containing the cDNA fragment (amino acid residues 2751 to 3041, Genbank Q62059) used for the generation of the versican GAG-β antibody, was kindly provided by M.T. Dours-Zimmermann. This vector was used to generate the recombinant peptide used as antigen.

2.2.1. Preparation of Antigens

E.Coli M15[pREP4] cells were used to express the His-tagged fusion proteins. Recombinant peptides were purified on Ni²⁺-NTA agarose (Qiagen) columns. The protocol suggested by the supplier for the purification of fusion proteins under denaturing conditions was followed. Briefly, after extraction with Buffer A (6M guanidinium hydrochloride, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8) for 15 min, the cleared lysate was mixed with the Ni²⁺-NTA agarose resin by gentle stirring for 2 h. Following assembling, the column was extensively washed with Buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8) and elution was achieved by applying a 6.5-4.0 pH gradient in 8M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl. The fractions were analyzed on 4-25% PHAST gels stained with Coomassie blue.

2.2.2. Generation of Polyclonal Antibodies

Polyclonal antibodies were prepared at the Institute for Animal Science of the University Züriich. Rabbits were immunized with 30-400 µg soluble antigens emulsified in Freund incomplete adjuvant. After 4 boosts rabbits were sacrificed and immunogenic antisera collected. The antisera against the GAG-α and the GAG-β domain of versican were
preadsorbed on bacterial extract columns followed by purification on the corresponding fusion protein columns. All affinity columns were prepared as described below.

2.2.3. Affinity Purification

For the preparation of the fusion protein or the bacterial extract columns, purified fusion peptides or extracts of mock transformed bacteria were immobilized on 5 ml NHS-activated HiTrap columns (Pharmacia, Uppsala, Sweden), according to the supplier's instructions. Briefly, protein solutions extensively dialyzed against coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3), were covalently linked to the column by recirculation for 3 h at room temperature using a peristaltic pump. After blocking with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 columns were washed with alternating high/low pH buffers. The affinity columns were kept at 4°C in PBS/0.02% NaN₃.

2.3. Immunohistochemistry

Mice were euthanized with CO2 and dissected. Collected tissues were fixed overnight with 4% paraformaldehyde in PBS. After dehydrating and paraffin embedding, 2-5 µm thick paraffin sections were cut on a microtome and mounted on SuperFrost Plus glass slides (Menzel Gläser) for immunohistochemical processing. Paraffin sections were dewaxed in xylole and rehydrated in a descending ethanol series. To improve immunostaining antigens were retrieved by steam-cooking the slides for 2 minutes in TEC buffer (2 mM Tris, 1.7 mM EDTA, 1 mM tri-sodium citrate, pH 7.8). After antigen retrieval sections were blocked with 3 % normal donkey serum in PBS for 30 minutes, and incubated overnight with appropriate primary antibodies in blocking buffer. Following primary antibody dilutions were used: anti-GAG-β pAb 1:1000 (versican V0/V1); anti-GAG-α pAb 1:1000 (versican V0/V2). Following extensive washing steps in PBS, sections were incubated with a biotinylated secondary anti-rabbit antibody (Biotin-SP-conjugated AffiniPure Donkey Anti-Rabbit IgG, 1:1000, Jackson ImmunoResearch). Endogenous peroxidase activity was quenched by incubation with 0.3 % H₂O₂ for 30 minutes. After additional washes in PBS sections were incubated with a peroxidase-conjugated avidin-biotin complex (ABC-kit, Dako), washed and visualized with a 0.06 % DAB solution followed by counterstaining. Finally sections were mounted in glycerol.
3. Results

3.1. Sequencing of the Target Region

Since at the beginning of this project the complete sequence of the mouse genome was not yet known and only the promoter and exon sequences of the versican gene were of public domain, several PCR approaches were used to clone fragments covering the target region (Figure 3.1). A long distance PCR using primers that hybridize in exon I and II, was performed to amplify a fragment of 10.8 kb containing the entire intron I of the versican gene. An inverted PCR approach was used to amplify fragments from upstream of the promoter region. After two successive rounds of inverted long distance we obtained three overlapping fragments of 3.2 (PstI fragment), 2 (HindIII fragment) and 6.5 kb (EcoRI fragment), respectively. After subcloning these PCR-fragments we determined the sequence by primer walking or by subcloning smaller restriction fragments into a pBluescript KS (+) vector followed by sequencing with M13 forward and reverse primers. This way we obtained a continuous sequence of about 20 kb covering the entire region of the versican gene chosen for gene targeting. Starting from this sequence we positioned the loxP sites, designed primers for the amplification of the three components (5' arm, promoter/exon I and 3' arm, respectively) of the targeting construct and we planned the Southern blot and PCR strategies used to screen the ES cell clones for homologous recombination.

**Targetin region**

![Diagram of the target region with restriction enzymes and primers](image)

**Figure 3.1:** PCR-strategies used to clone and sequence the targeting region. The intron I of the versican gene was amplified by long distance PCR using primers annealing to the exon I and II, respectively. Inverse PCR was performed using the primer pairs shown in the scheme. Restriction enzyme and primer selection for this inverse PCR was based on the results of a Southern blot analysis. Following digestion, genomic DNA fragments were circularized by ligation. Primers for the second inverse PCRs were designed on the basis of the newly obtained sequences from the first round. All the PCR amplified fragments were subcloned and sequenced.
3.2. Generation of the Targeting Constructs

Based on sequence alignment of the human and mouse versican promoter, we decided to flox a conserved 700 bp region of the mouse genome, which include all the putative regulatory elements described by Naso and colleagues for the human versican promoter. Figure 3.2 shows the sequence homology between the human and mouse versican promoter and the precise site of insertion of the loxP sequences.

Figure 3.2: Sequence analysis showing the homology between the human (lower sequence) and mouse (upper sequence) versican promoter and the insertion sites of the loxP sequences. The putative regulatory elements, including the TATA box, XRE, CCAAT-enhancer binding protein (C/EBP), AP2, SP1, CTF/CBF, CBP and cyclic AMP-responsive element-binding protein (CREB) are underlined. The additional sequences in the mouse promoter show the location of the two loxP sites (red) inserted for the conditional inactivation of the versican gene. Violet background marks the exon I sequence.
In order to inactivate the versican gene in a conditional manner we designed a targeting construct that upon integration by homologous recombination and subsequent removal of the selection cassette by transient Cre expression, would leave in the targeted allele two loxP sites flanking the versican promoter. Separate targeting constructs were prepared for two genetic backgrounds (129/Sv and C57BL/6 strains) since the use of DNA isogenic with the ES cells has been reported to improve targeting efficiency. The components of the constructs, namely the 5' arm, the promoter/exon I fragment and the 3' arm, were obtained by PCR cloning using primers carrying appropriate restriction sites. The three fragments were assembled in a vector containing a floxed neo-tk cassette. A stepwise cloning strategy was used allowing the insertion of an additional loxP site in the middle of exon I. For a detailed description of the cloning strategy refer to the methods section. The constructs used to target the ES cells contain a floxed selection cassette followed by a 700 bp fragment corresponding to the versican promoter and part of the exon I in which a third loxP site was inserted. To facilitate homologous integration two additional regions of the versican gene were included, a 5'-arm of 4 kb and a 3'-arm of 5 kb length. The selection cassette encoding for the neomycin phosphotransferase gene (neo) was used for positive selection of stable transfected clones whereas the thymidine kinase gene (tk) was used for negative selection after transient Cre expression in the ES cells.

Figure 3.3: The conditional construct used for targeting. Partial restriction map of the Versican locus and schematic representation of the targeting construct and the targeted locus. Homologous recombination will result in the integration of a floxed neo-tk cassette upstream of the versican promoter (P) and a loxP site (red arrowhead) into the exon I (E I). After transient expression of Cre in ES cells one result is the removal of the neo-tk cassette, leaving a floxed promoter, which can be removed by a second round of Cre expression in vivo.

3.3. PCR and Southern Strategies for Screening of the ES Cells

Using the previously determined sequence of the versican promoter region, we designed Southern blot and PCR strategies for the screening of ES clones. After confirmation of the efficiency of our PCRs in detecting positive clones from the first round of ES cell screening, we used subsequently Southern blotting only to verify the results obtained by PCR and to investigate whether the integration event was complete and unique.
Figure 3.4 indicates the primer pairs used for the screening and characterization of the targeted ES cell clones. A primer located upstream to the targeting region and a primer unique to the targeting construct (Figure 3.4: 5’ up and 5’ low primers), were used in long distance PCR (LD-PCR). The resulting fragment was 4.8 kb in length. A second LD-PCR was designed as control (Figure 3.4: 3’ up and 3’ low primers) using upper and lower primers hybridizing in the neo-tk cassette and in the 3’ external region respectively. Using this primer pair, homologous recombined clones gave rise to a 7.2 kb-long product.

Homologous recombination was finally confirmed by Southern blotting using two different probes. BamHI digested genomic DNA was hybridized with a 5’ external probe (Figure 3.4: BamHI Probe) giving rise to a 6.9 kb band for the wild type and a 4.75 kb band for the mutant allele. Integrity of the 3’ region was confirmed using the Eco RV restriction enzyme in combination with a 3’ external probe (Figure 3.4: EcoRV Probe) that gave rise to 10.5 or 9.5 kb long bands for the wild type or mutated allele, respectively. Unique integration events were confirmed using SpeI digested genomic DNA hybridized with a probe located within the neo-tk cassette (Figure 3.4: NT-Probe). Single integration of the targeting construct by homologous recombination resulted in a unique band of 10.8 kb.

### PCR

<table>
<thead>
<tr>
<th>PCR</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ control</td>
<td>3’ control</td>
</tr>
<tr>
<td>(5’ up / 5’ low)</td>
<td>(3’ up / 3’ low)</td>
</tr>
<tr>
<td>wild type allele</td>
<td>---</td>
</tr>
<tr>
<td>targeted allele</td>
<td>4.8 kb</td>
</tr>
</tbody>
</table>

![Wild type allele diagram](image)

![Targeted allele diagram](image)

Figure 3.4: PCR and Southern strategies used to screen and characterize the ES cell clones after gene targeting.

Transient Cre expression in homologous recombined clones could lead to three different types of deletion (Figure 3.5). In practice, clones still containing the neo-tk cassette (homologous recombined and type III clones) were eliminated by GANC selection, whereas type I and type II clones were discriminated by PCR screening. For this purpose, we used a primer pair flanking the selection cassette (Figure 3.5: P1/2 up and P1/2 low primers). The resulting fragments were 159 and 295 bp in length for the wild type and the type II alleles, respectively. As control a second pair of primers (Figure 3.5: P3 up and P3 low) was used. In this case the wild type and the type II alleles gave rise to PCR products of 201 and 245 bp in length. To further characterize the positive clones, containing a type II allele, we used the P1/2 up and P3 low primers to PCR amplify and sequence the floxed versican promoter.
Finally, correct deletion of the selection cassette was confirmed by Southern blotting using BamHI digested genomic DNA and a probe located in the intron I of the versican gene (Figure 3.5: Probe A). The wild type allele resulted in a band of 6.9 kb size whereas the type II allele yielded a 2.3 kb fragment.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Southern</th>
</tr>
</thead>
<tbody>
<tr>
<td>loxP1/2</td>
<td>loxP3</td>
</tr>
<tr>
<td>(P1/2 up / P1/2 low)</td>
<td>(P3 up / P3 low)</td>
</tr>
<tr>
<td>wild type allele</td>
<td>159 bp</td>
</tr>
<tr>
<td>targeted allele</td>
<td>3420 bp</td>
</tr>
<tr>
<td>type I allele</td>
<td>--</td>
</tr>
<tr>
<td>type II allele</td>
<td>295</td>
</tr>
<tr>
<td>type III allele</td>
<td>--</td>
</tr>
</tbody>
</table>

Figure 3.5: PCR and Southern strategies used to screen and characterize the ES cell clones after transient Cre transfection.

3.4. Generation and Characterization of Targeted Clones

A crucial event in the generation of genetically modified mice using the gene targeting technology in ES cells is the capability of the ES cells to contribute to germ line transmission. In particular, prolonged in vitro culture of the ES cells may lead to partial differentiation or accumulation of chromosomal aberrations thus reducing the pluripotency of the ES cells. In order to obtain homologous recombined ES clones, which were able to contribute to germ line transmission, we had to repeat ES cells targeting experiments several times. Table 3.1 summarizes the results of the different targeting experiments performed with the Bl/6-III and R1 ES cell lines.
Table 3.1: Summary of the targeting experiments performed to generate type II ES cell clones able to contribute to germ line transmission. FCS = fetal calf serum; KSR = Knockout serum replacement; (*) ES cell line containing a translocation; (") clones lacking the third loxP site

<table>
<thead>
<tr>
<th>ES-cell line</th>
<th>Gene targeting</th>
<th>Transient Cre expression</th>
<th>Blastocyst injected clones</th>
<th>Germline transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homologous recombined clones / total nr. picked</td>
<td>Clone Nr.</td>
<td>Type II deleted clones / total nr. picked</td>
<td></td>
</tr>
<tr>
<td>Bi/6-III-FCS*</td>
<td>2/288 (0.69 %)</td>
<td>Bi/6-III-FCS-37</td>
<td>4/192 (2.08 %)</td>
<td>Bi/6-III-37-20&lt;br&gt;Bi/6-III-37-42&lt;br&gt;Bi/6-III-37-77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bi/6-III-FCS-94</td>
<td>3/96 (3.13 %)</td>
<td></td>
</tr>
<tr>
<td>Bi/6-III-KSR*</td>
<td>3/288 (1.04 %)</td>
<td>Bi/6-III-KSR-395</td>
<td>3/192 (1.56 %)</td>
<td>Bi/6-III-KSR-395-32&lt;br&gt;+ 10 subclones</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bi/6-III-KSR-563&lt;br&gt;Bi/6-III-KSR-573</td>
<td>3/96 (3.13 %)&lt;br&gt;5/96 (5.2 %)</td>
<td></td>
</tr>
<tr>
<td>Bi/6-III-KSR</td>
<td>1/576 (0.17 %)</td>
<td>Bi/6-III-KSR-562</td>
<td>19/384 (4.95 %)</td>
<td>Bi/6-III-KSR-562-5&lt;br&gt;Bi/6-III-KSR-562-20&lt;br&gt;Bi/6-III-KSR-562-31&lt;br&gt;Bi/6-III-KSR-562-93&lt;br&gt;Bi/6-III-KSR-562-164&lt;br&gt;Bi/6-III-KSR-562-155</td>
</tr>
<tr>
<td>R1-FCS</td>
<td>3/384 (0.78 %)</td>
<td>R1-FCS-70*&lt;br&gt;R1-FCS-269*&lt;br&gt;R1-FCS-290*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1-FCS</td>
<td>2/576 (0.35 %)</td>
<td>R1-FCS-235&lt;br&gt;R1-FCS-259</td>
<td>31/384 (8.07 %)</td>
<td></td>
</tr>
</tbody>
</table>

In a first attempt we generated several type II clones using a Bi/6-III ES cell line. Animals with only a low percentage of chimaerism could be obtained by injecting these ES cells into blastocysts, even using subclones of the distinct floxed clones. Finally it was found that the parental ES cell line contained already at the beginning of the targeting experiments a translocation involving two different chromosomes. This abnormality probably caused the loss of germ line competence.

Subsequently a new batch of Bi/6-III ES cells, tested to have a normal karyotype, was electroporated with the linearized targeting vector. ES cells were grown in selection culture medium to allow those cells to survive that contained the neomycin gene, thereby selecting for ES cells that harbor the targeting construct. From the plates, 576 clones were picked and further processed. For the first round of PCR screening, the genomic DNAs of eight clones were pooled in order to reduce the number of PCR analyses. One pool gave rise to a band of the expected size (Figure 3.6 A). Reanalysis of single clones revealed that clone Bi/6-III-KSR-562 had integrated the targeting vector in the versican locus (Figure 3.6 B).
Figure 3.6: PCR screening of the transfected ES cell clones. PCR screening was performed using pools representing the DNA of eight ES cell clones followed by individual analyses of the single clones from the positive pools. (A) Bl/6-III ESC line. (B) Rl ESC line.

Figure 3.7: PCR and Southern analyses of the homologous recombined clones. M, molecular weight marker; 1, Bl/6-III (wild type); 2, clone Bl/6-III-KSR-562; 3, R1 (wild type); 4, clone R1-FCS-259.

The correct integration of the targeting construct in clone Bl/6-III-KSR-562 was confirmed by additional PCR and Southern blot analyses (Figure 3.7). Clone Bl/6-III-KSR-562 was then expanded and transfected with a vector for transient Cre expression. After electroporation ES cells were grown in culture medium supplemented with gancyclovir in order to select for clones, in which the Cre recombinase had deleted the DNA portion containing the neo-tk cassette. PCR analysis of 384 clones revealed that 19 of them had undergone a suitable deletion (type II deletion efficiency of 4.9 %). Six of these positive clones were further analyzed by PCR and Southern blotting (Figure 3.8), expanded and finally stored in aliquots in liquid nitrogen until injection.
The strategy used to generate type II R1 clones was basically the same as for the Bl/6-III cell line. Also in this case targeting had to be repeated twice since in a first attempt to generate homologous recombinant clones, all the three positive clones had not integrated the third loxP site. Potential point mutations present in the 3' arm of the targeting construct could have been the cause for a homologous recombination occurring between the 5' arm and the versican promoter. Thus we decided to reamplify the 3'-arm using a minimal number of PCR cycles and to reassembly the 129/Sv targeting construct.

In the next trial 576 clones were picked after G418 selection and tested for targeted integration. In this way we identified 2 positive clones (targeting efficiency of 0.35 %). Further characterization of clones R1-FCS-235 and R1-FCS-259 revealed that indeed both clones had correctly integrated the targeting construct. Subsequently, these two clones were transiently transfected with the construct encoding the Cre recombinase. The clone R1-FCS-235 gave no type II positive clones, whereas 31 out of 384 type II positive clones were detected after transient transfection of clone R1-FCS-259 (efficiency of type II deletion of 8 %). Six of these subclones were further analyzed by PCR and Southern blotting. Finally clone R1-FCS-259-69 was used for injection into blastocysts.

### 3.5. Generation of the Versican Floxed Mice

Chimaeric mice were generated by injection of clones Bl/6-III-KSR-562-144 and R1-FCS-259-69 into CFVBF1 or C57BL/6 blastocysts, respectively. Both clones produced highly chimaeric animals (80-95%) as judged by the contribution of C57BL/6 or 129/Sv derived black or brown fur color. In order to maintain the animals in a pure C57BL/6 or 129/Sv genomic background, chimaeric animals were backcrossed with wild type C57BL/6 or 129/Sv genomic background, chimaeric animals were backcrossed with wild type C57BL/6 or 129/Sv...
mice, respectively. To test whether injected ES cells participated in germline formation, we tested all the offsprings by PCR for the presence of the mutated allele. Germline transmission of the floxed versican promoter was obtained from chimaeras of both genomic backgrounds. Finally to generate homozygous floxed mice, C57BL/6 and 129/Sv heterozygous F1-mice were inbred yielding wild type, heterozygous and homozygous mice in a normal Mendelian ratio. The integrity of the targeted allele was verified by PCR and Southern blotting (Figure 3.9). Both, the heterozygous (Csgp2 \textsuperscript{floxed/+}) and homozygous (Csgp2 \textsuperscript{floxed/floxed}) mice were viable and fertile, and did not display any obvious phenotype.

**Figure 3.9:** PCR and Southern blot analyses of heterozygous and homozygous floxed mice. M, molecular weight marker; 1, wild type C57BL/6; 2, heterozygous floxed C57BL/6; 3, homozygous floxed C57BL/6; 4, parental ESC clone B1/6-II-KSR-562-144 (type II, floxed); 5, wild type 129/Sv; 6, heterozygous floxed 129/Sv; 7 homozygous floxed 129/Sv; 8 parental ESC clone R1-FCS-259-69 (type II, floxed).

Immunohistochemical analysis using antibodies against the GAG-\(\alpha\) or GAGA-\(\beta\) domain of the versican core protein, revealed in heterozygous and homozygous mice no differences in distribution of the large proteoglycan isoforms in comparison to their wild type littermates. (Figure 3.10; 3.11). Using the GAG-\(\alpha\) antibody, we observed in both, heterozygous and homozygous adult animals the typical versican V2 staining in the white matter of the cerebellum with especially strong color deposition at the node of Ranvier, (Figure 3.10). Similarly we could detect an apparently normal expression of versican V0/V1 in the heart valves of Csgp2 \textsuperscript{floxed/+} and Csgp2 \textsuperscript{floxed/floxed} (Figure 3.11). Since normal distributions of versican V0, V1 and V2 were revealed by our immunohistochemical analyses, we concluded that the insertion of the two loxP sites flanking the versican promoter did not interfere with the normal versican expression.
Figure 3.10: The expression of versican V2 in the white matter of the cerebellum is not impaired in the heterozygous and homozygous floxed mice.

Figure 3.11: Normal expression of versican V1 in the valves of the heart of heterozygous and homozygous floxed mice.
4. Discussion

4.1. Why a Conditional K.O. for Versican?

Versican is a chondroitin sulfate proteoglycan belonging to the family of hyalectans. All hyalectans are expressed in the CNS with a unique pattern. CSPG are in general believed to inhibit axonal outgrowth and thus may modulate development of the neuronal circuits during embryogenesis and to restrict the structural plasticity in the adult brain. There is increasing evidence that versican might be involved in the development and stabilization of the central and peripheral nervous system by acting as negative modulator of cell migration and axonal outgrowth. In particular versicans V0/V1 are suggested to have roles in cell migration and axonal guidance during brain development, whereas versican V2 appears to be involved in limiting structural plasticity in the adult mammalian CNS. However, these notions about versican functions are currently still based on indirect evidence accumulating from in vitro experiments. Despite of the existence of a constitutive versican knockout strain, an in vivo confirmation of the putative versican functions in the nervous system development is still missing. Mjaatvedt et al. generated this versican knockout strain by an accidental transgene insertion within the Cspg2 locus (Mjaatvedt et al., 1998). This mutant strain was termed heart defect (hdf), since embryos homozygous for the transgene insertion die relatively early in utero due to a defect in the development of the heart. Thus the hdf mouse model does not provide the opportunity to study versican functions at later embryonic stages or in the adult organism. Consequently, the mayor aim of this work was to develop a murine model, in which all the versican isoforms could be inactivated in a conditional manner, thus overcoming the embryonic lethality found in the hdf mice.

4.2. Strategy for Creating Versican Conditional K.O. mice

To circumvent the embryonic lethality associated with a constitutive versican gene knockout, we took advantage of the cre/lox recombination system. This approach involves the generation of a mouse strain with a floxed conditional allele, which should be completely functional, followed by in vivo inactivation in a cell type specific and temporally controlled fashion by cross-breeding with mice transgenic for the Cre recombinase. Since the versican gene covers more than 100 kb on the mouse chromosome 13, we had to identify a functionally indispensable gene element for the knock-in of two flanking loxP sites. The following criteria had to be fulfilled: 1) removal of the floxed region should lead to the successful abrogation of expression of all four versican isoforms; 2) the normal versican expression should not be impaired by integrating the loxP sequences into crucial regulatory elements e.g. in the not yet characterized first intron; 3) an expression of artificial truncated core proteins caused by alternative translation initiation or early termination should be avoided; 4) the floxed sequence should be relatively short to permit a single targeting approach keeping the risk for unwanted in vitro differentiation of ES clones low and to allow efficient in vivo excision by Cre recombinase. Following this selection scheme, we finally decided to target the 5'-promoter region. Functionality of this region had been previously demonstrated for the humane homologue sequence by in vitro studies characterizing the regulatory elements through deletion
experiments (Naso et al., 1994) and by in vivo studies using the human versican promoter to drive lacZ and GFP reporter gene expression in transgenic mice (Kishimoto et al., 1999). The more conventional and probably safer strategies to flox the entire versican gene, or to position one loxP site upstream of the promoter and one downstream of the ATG translational START codon were discarded. This is due to the fact that the versican gene spreads over more than 100 kb, and the first ATG codon is located in the second exon of the versican gene residing more than 10 kb downstream of its promoter. Thus these strategies would have required two targeting events with a total of four electroporations, making it unlikely that the final ES clones would have been still pluripotent.

In our approach, we used a tri-lox strategy, in which a floxed selection cassette was introduced upstream of the versican promoter and a third loxP site had been placed into exon I leaving the first intron untouched. After successful targeting, the selection marker could be removed by transient Cre-expression in the ES cells, thus avoiding a possible interference with the expression of the targeted or the neighboring genes. Despite of the fact that this strategy is routinely used it has some disadvantages. In particular, the prolonged culture of ES cells may result in the loss of pluripotency and/or promote the accumulation of chromosomal aberrations. Furthermore the ES cells have to be screened again after Cre transient expression to identify clones, in which only the selection cassette has been removed. One alternative to overcome these problems would have been the in vivo removal of the selection cassette using a Cre balancer mouse. However in this approach a large number of double transgenic mice have to be generated to obtain an optimal level of mosaicism for segregation breeding (Leneuve et al., 2003). Another more elegant option would have been to flank the selection marker with FRT sites and the versican promoter with loxP sites. This way the selection cassette could have been removed in vivo by crossing mice bearing the targeted allele with a Flp deleter transgenic line (Farley et al., 2000; Rodriguez et al., 2000). This second approach minimizes the manipulation of ES cells, and avoids selection for the correct type of deletion. However, efficient Flp deleter mice were not available at the beginning of this project. In both of these in vivo excision approaches the thymidine kinase gene, one of our selection markers, would have to be removed from the construct, as its expression interferes with male fertility.

Since the use of DNA isogenic with the ES cells used for targeting has been reported to improve targeting efficiency, we prepared vectors for the 129/Sv and C57BL/6 genetic backgrounds. The three loxP sites were inserted in a “reverse” orientation. In fact, the “forward” loxP sequence contains the TATAAT sequence similar to the TATA-Box of the versican gene, which could potentially lead to a leaky expression even after excision of the versican promoter. The homologous elements of the constructs (5’ arm, the promoter/exon I, and the 3’ arm fragments) were generated by PCR. Compared to the classical method based on the screening of genomic DNA libraries, the PCR approach is faster and using primers carrying exogenous restriction sites we could choose the exact site of insertion of the loxP sequences. However PCR amplification could generate single base pair mutations, which may reduce targeting efficiency. To minimize that we used a Taq polymerase mixture with proof reading system and we kept the number of amplification cycles as low as possible.

4.3. Choice of Embryonic Stem Cells

Since most of the Cre transgenic lines have been generated on a C57BL/6 genomic background, we initially decided to use the Bl/6-III cell line for our targeting experiments. Although this line is known to transmit to germline with low efficiency, it offers several advantages. Firstly it is possible to directly generate mice with C57BL/6 genomic background, which is usually also the background of the Cre-expressing transgenic strains. A
time-consuming backcrossing scheme is therefore not required to obtain a well defined inbreed strain. Secondly the Bl/6-III cell line has been adapted to growth in serum replacement supplemented media. Unlike FCS, which may contain undefined factors promoting ESC differentiation, serum replacement is a defined formulation, thus time and cost consuming screening of different serum batches can be avoided. Finally, ES cells grown in serum replacement appear substantially less differentiated than those grown in FCS supplemented media.

However due to the delay in obtaining germ line transmission mice with Bl/6-III ES cells, we decided to use R1 ES cells as well, since they are widely known for their high efficiency for germline transmission.

4.4. Generation of Versican Floxed Mice (Cspg2^{floxed/floxed})

As already mentioned in the results section the generation of floxed ESC clones has been particular difficult due to a series of technical problems linked to the ESC line used for gene targeting. Since the initially used passage of these C57BL/6 derived ES cells contained already at the beginning a translocation, which had before not been noticed, the gene targeting did not lead to a germline transmission in the first round. The second attempt using an earlier batch without the genetic aberration was then successful. Similarly we had to repeat the targeting with the R1 ES cells, too. In the first round we could obtain only clones that had only partly integrated the modified promoter lacking the third loxP site. Finally however, we obtained chimaeras that gave germline transmission from both, the BL6-III and the R1 clones. Gene targeting was done using constructs isogenic for the Bl/6-III or the R1 ESC lines, however targeting efficiency was quite low with both lines (Bl/6-III: 0.17 %; R1: 0.35 %). Possible reason for such a low targeting efficiency could be the target locus itself and its transcriptional activity, or putative point mutations present in the 5' and 3' arm of the targeting construct generated during PCR amplification. In vitro removal of the selection cassette was achieved for both ESC lines, with a slight higher efficiency for the R1 (8 %) than for the Bl/6-III (5 %) ESC.

Blastocyst injection of clones Bl/6-III-KSR-562-144 and R1-FCS-259-69 was done at the institute of laboratory animal science (LTK, University of Zurich), or at the department of molecular medicine (Max Planck Institute for Biochemistry, Martinsried, Germany). The generation of floxed mice with 129/Sv background has been in general more efficient than with the C57BL/6 background. From one blastocyst injection experiment, using clone R1-FCS-259-69, we obtained six chimaeric males, one of which was able to germline transmission with an estimated efficiency of 66 %. While several trials were needed to obtain viable chimaeric animals from injection of clone Bl/6-III-KSR-562-144. A temporary mouse parvovirus (MPV) outbreak at the animal facility of the LTK was probably detrimental to the generation of chimaeric mice. In fact MPV is known to induce spontaneous abortion and stillbirth in mice. In sum, both the Bl/6-III and 129/Sv ESC lines were in our hands suitable for the generation of genetically modified mice. Generation of targeted mice using R1 cells has been generally faster and more efficient. However a definitive comparison of the efficiency of the two ESC lines can not be deduced from our experiments, since the blastocyst injections were performed at two different places under different conditions. From the two chimaeric animals that transmitted the floxed promoter into germline, we generated mouse lines for the C57BL/6 or the 129/Sv genomic background. Both lines displayed normal sex ratios, survival, and behavior and did not display any obvious phenotype. The generation of floxed mice (Cspg2^{floxed/floxed}) was confirmed at the genomic level by PCR analysis and Southern blotting of genomic DNA from mouse tail biopsies.
Normal expression of the different versican isoforms, was demonstrated by immunohistochemical analysis of brain and heart sections suggesting that the activity of the versican promoter is not impaired by the presence of the loxP sites.
5. Outlook

5.1. Analysis of the Csg2 deleted/deleted Embryos

In order to test the effectiveness of our versican gene inactivation approach and to verify the embryonic lethal phenotype of the hdf mutants, we have generated a mouse strain with a versican promoter allele deleted in the germinal cells. This was done by crossing heterozygous floxed mice (Csg2floxed/+ ) with N6Del mice (Schwenk et al., 1995). These latter mice express Cre under the control of the strong CMV promoter very early in development. Consequently the floxed versican promoter allele was constitutively removed in the double transgenic mice. The Csg2deleted/+ mice are being intercrossed to obtain homozygous deleted (Csg2deleted/deleted) animals. If we can confirm abrogation of the versican expression we will analyze the phenotype of the Csg2deleted/deleted mouse line in more detail. For example we want to study whether neural crest cell (NCC) migration is impaired in the Csg2deleted/deleted animals. In fact we previously showed versican expression in the caudal half of the somitic sclerotome, which is avoided by the migrating NCC (Landolt et al., 1995), furthermore we recently demonstrated that versican is a non permissive substrate for migration of NCC in vitro (S. Dutt-Raghav, manuscript in preparation).

5.2. Generation of Nervous Tissue Specific Versican Conditional K.O. Mice

Generation of additional conditional knockout mice by crossing the Csg2floxed/floxed strain with other transgenic mouse lines that specifically express Cre in the developing or the adult central nervous system, will provide us valuable mouse models to study the role of versican in brain development, function and disease. We are currently crossing the Csg2floxed/floxed mice with two different nervous tissue specific Cre transgenic mouse lines: In the first strain, Cre expression is driven by the rat nestin promoter and the nervous system specific enhancer present in the second intron of the rat nestin gene (Tronche et al, 1999). Nestin is an intermediate filament gene that is a marker for neural stem cells of developing and adult brain. Troche and colleagues, used the same Nestin-Cre strain to successfully abrogate expression of the glucocorticoid receptor in the nervous system (Tronche et al., 1999).

Using this Nestin-Cre mouse strain, we expect to delete the versican promoter in neuronal and glial precursor cells, and thus abrogate expression of all versican isoforms in the developing and adult nervous system. In particular we will use this mouse (Csg2 floxed/floxed; NesCre/+ ) to understand, whether ablation of versican expression in the nervous tissue has an effect on the tangential migration of cortical and olfactory interneurons. In fact we have noticed that, during embryogenesis versican V0/V1 is transiently expressed in the cortical plate of the developing brain, which is usually avoided by neuronal precursor cells during their journey from the ganglionic eminences to the cerebral cortex, where they differentiate to cortical interneurons (Figure 5.1). Furthermore we have recently found that in the adult mouse brain expression of versican V0/V1 correlates with the rostral migratory stream (Figure 5.2), which is the pathway used by adult neuronal stem cells, generated in the subventricular zone of the forebrain, on their way to the olfactory bulb. Similarly in the subventricular zone, versican V0/V1 is present in the basal lamina surrounding the germinal centers were neurogenesis occurs (Figure 5.3). In this case we hypothesize that versican, probably expressed by the
astrocytes ensheathing the migrating neuroblasts, might prevent that neuronal precursor cells leave the RMS precociously. Currently, we have generated Cspg2\textsuperscript{floxed/+}; NesCre\textsuperscript{0} mice and have crossed them with Cspg2\textsuperscript{floxed/floxed} mice, this way we obtained a conditional versican K.O. (Cspg2\textsuperscript{floxed/floxed; NesCre\textsuperscript{0}}) mouse strain carrying the hemizygous Cre allele.

Figure 5.1: (A) Scheme of a section through the developing mouse forebrain. During their journey from the ganglionic eminence (GE) to the neocortex, migrating interneurons (blue and pink cells) avoid the cortical plate region (CP; blue band), which is suggested to contain repellent signals. (B) Expression of versican V0/V1 in the cortical plate of the mouse embryonic brain.

Figure 5.2: (A) Schematic sagittal view of the adult mouse brain showing the exposed lateral wall of the lateral ventricle (shaded area) and migratory pathways of neuronal precursors to the olfactory bulb. CB: cerebellum; cc: corpus callosum; M: foramen of Monro; NC: neocortex; OB: olfactory bulb; RMS: rostral migratory stream. (B) Anti-GAG\beta staining reveals the presence of versican V0/V1 along the adult mouse RMS.
Figure 5.3: (A) Schematic coronal view of the adult mouse brain illustrating the localization of the subventricular zone (SVZ). The adult SVZ consists of chains of neuroblasts (type A cells, red) ensheathed by SVZ astrocytes (type B cells, blue). Adjacent to these chains are immature precursors (type C cells, green). Ependymal cells (grey) form an epithelial layer separating the SVZ from the ventricle. LV: lateral ventricle; BL: basal lamina; BV: blood vessel (Alvarez-Buylla and Lim, 2004). (B) Expression of versican V0/V1 in the adult mouse brain SVZ correlate with the germinal centers where new neurons are generated.

A second conditional strain will be generated using the En2-cre transgenic strain (Zinyk et al., 1998). In this case Cre recombinase expression is under the control of the engrailed 2 (En2) embryonic enhancer, and the En2 minimal promoter fragment. En2-cre mice have been reported to drive Cre expression in the midbrain-hindbrain constriction at around E 9.5. Thus using this Cre transgenic mouse we should be able to restrict the inhibition of versican expression to cerebellum. This will help us to investigate the functions of versican in the migration of pyramidal and cerebellar cortical neurons.
PART II: Functional Analysis of Versican V2 Through ADAMTS Mediated Proteolysis

1. Introduction

One of the most fascinating and critical events in brain development is the establishment of correct neuronal connections, which is crucial for proper functioning of the vertebrate nervous system. After CNS injury and diseases a similarly precise re-wiring of neuronal connection would be required for functional recovery. Unfortunately this regeneration process is greatly impaired by the axon growth inhibitory properties of the mature CNS. During development of the nervous system, neurons project axons over long distances in order to reach their final targets. Along the way, growth cones located at the leading edge of axons sense and respond to environmental cues that guide them to their appropriate destinations.

An increasing number of families of guidance molecules, conveying attractive and/or repulsive signals to the growth cone, have now been identified. Some of these molecules are diffusible factors providing long-range cues, others are non diffusible cell surface or extracellular matrix proteins that act over short distances via cell-cell or cell matrix contacts. Whereas combination of molecules like netrins, neurophilins, semaphorins, ephrins, Eph receptors, IgCAMs, chadherins and neurotrophins (Tessier-Lavigne and Goodman, 1996; Mark et al., 1997; Cook et al., 1998; Flanagan and Vanderhaeghen, 1998; Holt and Harris, 1998; Stocekli and Landmesser, 1998; Uemura, 1998) may provide a fine-tuning mechanism to guide individual growth cones to their appropriate target, extracellular matrix proteins like laminins, fibronectins and proteoglycans (Hynes and Lander, 1992; Margolis and Margolis, 1997; Bandtlow and Zimmermann, 2000) may contribute to axonal navigation by forming permissive pathways or barriers to the extending axon.

Increasing evidence indicates that chondroitin sulfate proteoglycans (CSPGs) may act as inhibitors of axonal extension during nervous tissues development as well as in pathological conditions. In general CSPGs, are believed to interfere with cell-matrix and cell-cell interactions, thus are considered to be negative modulators of cell and axon adhesion (Grumet et al., 1993; Grumet et al., 1996; Oohira et al., 2000). Expression analysis demonstrate that during embryonic development CSPGs are present in tissues that form barriers to the migrating neural crest cells and elongating axons of the central and peripheral nervous system (Oakley and Tosney, 1991; Bronner-Fraser, 1994; Landolt et al., 1995; Fitch and Silver, 1997). In vitro inhibitory activity on neurite outgrowth has been demonstrated for several CSPGs, like neurocan (Friedlander et al., 1994), brevican (Yamada et al., 1997), phosphacan (Milev et al., 1994), aggrecan (Snow et al., 1990), versican (Schmalfeldt et al., 2000) as well as the cell surface proteoglycan NG-2 (Dou and Levine, 1994). Furthermore CSPGs are inhibitory components of the glial scar that forms after a CNS injury (Fawcett and Asher, 1999; Asher et al., 2001). An increased expression following CNS injury was detected for NG2 (Levine, 1994; Fidler et al., 1999) (Jones et al., 2002), phosphacan (McKeon et al., 1999; Jones et al., 2003), neurocan (Haas et al., 1999; Asher et al., 2000; Jones et al., 2003), brevican (Jaworski et al., 1999; Thon et al., 2000; Jones et al., 2003) and versican (Asher et al., 2002; Jones et al., 2003; Tang et al., 2003).

In particular the hyalectans (or lecticans) (Ruoslhti, 1996; Iozzo, 1998) have gained growing attention in regard to their putative functions in axonal guidance during development (Margolis and Margolis, 1993; Margolis and Margolis, 1997; Bandtlow and Zimmermann, 2000), in structural stabilization of the mature central nervous system (Hockfield et al., 1990;
Yamada et al., 1997), and in inhibiting regeneration in the lesioned CNS (Fawcett and Asher, 1999; Sandvig et al., 2004). Currently the hyalectan CSPG family includes aggrecan (Doege et al., 1987), brevican (Yamada et al., 1994), neurocan (Rauch et al., 1992) and versican (Zimmermann and Ruoslahti, 1989). All hyalectans have highly conserved N- and C-terminal globular domains, which mediate interaction with hyaluronan and tenascins, respectively (Iozzo, 1998; Bandtlow and Zimmermann, 2000). In contrast the intervening regions to which CS side chains may be attached are highly diverse between the different hyalectans. In the versican gene alternative splicing of the two large exons (GAG-α, and GAG-β) encoding for the chondroitin sulfate carrying middle portions of the core protein give rise to at least four different versican isoforms, namely versican V0, V1, V2, and V3 (Dours-Zimmermann and Zimmermann, 1994; Ito et al., 1995; Zako et al., 1997).

The versican V2 isoform, which contains only the GAG-α domain, has been suggested to play a significant role in suppressing excessive axonal growth in the adult CNS and in inhibiting regeneration following injury. Isolated versican V2 has been shown to be a potent inhibitor of neurite outgrowth of central and peripheral neurons, in vitro (Schmalfeldt et al., 2000). In vivo, versican V2 forms part of the central nervous system myelin, which is known for its axon growth inhibitory properties (Niederost et al., 1999). The late onset of the expression of versican V2 correlates with the phase of myelination (Milev et al., 1998) and coincides with the reduction in plasticity and regenerative capacity of the maturing CNS.

Whereas versican V2 and brevican are the major hyalectans of the adult brain and spinal cord tissue, neurocan and the large versican isoforms V0 and V1 are expressed primarily during earlier phases of central nervous system development. Intact neurocan and of versican V0 and V1 are only transiently present. The large splice variants of versican practically disappear from CNS tissues at the end of the development. The rapid removal of the core proteins is linked to an efficient and specific degradation. Recently, several members of the ADAMTS protease family (a disintegrin and metalloproteinase with thrombospondin motif I) have been found to exhibit versicanase activity. Especially ADAMTS-1 -4 and -9 have been identified as potential ‘versicanases’ cleaving versicans at specific sites within the GAG-α and the GAG-β domains. (Sandy et al., 2001; Somerville et al., 2003; Westling et al., 2004) Using an antibody raised against the neo-epitope generated after in vitro ADAMTSs mediated cleavage of versican V2, it was demonstrated that the previously characterized GHAP (glial hyaluronate binding protein) is a product of ADAMTS mediated cleavage of versican V0 or V2, however the physiological significance of this cleavage remains to be clarified.

In this study, we examined whether the proteolytic activity of ADAMTS-4 may control the inhibitory activity of versicans and thus potentially modulates axonal growth during development and maturation of the nervous system.
2. Materials and Methods

2.1. Generation of Polyclonal Antibodies Against Recombinant Fragments of ADAMTS Endopeptidases

Degenerated primers of conserved sequences were designed on the basis of multiple alignments of mouse and human ADAMTS cDNA sequences. Total RNA was prepared from adult mouse brain or E-14.5 mouse embryos using RNaseasy Mini or Midi kits (Qiagen). The supplier’s instructions were followed. RT-PCR was performed with a OneStep RT-PCR kit (Qiagen) using two pairs of degenerated primers (ATS-UP1/ATS-LOW and ATS-UP2/ATS-LOW). The PCR-fragments containing putative ADAMTS encoding sequences were subcloned into the pGemTeasy vector. Inserts were sequenced and analyzed by BLAST (Basic local alignment search tool) search of a public genome database (http://www.ncbi.nlm.nih.gov), looking for regions with sequence similarity to previously described ADAMTS endopeptidases. Finally, PCR amplification with specific primers carrying the restriction sites Sfil and NotI provided fragments suitable for subcloning into a modified pDS9 bacterial expression vector (Zimmermann et al, 1994). This included cDNA fragments of ADAMTS-1 (amino acids: 390-542, Genbank: D67076), -4 (amino acid: 405-516, Genbank: NM_172845) and -5 (amino acid: 415-570, Genbank: AF140673). Table 2.1 summarizes all primer pairs and reaction conditions used to synthesize the ADAMTS cDNA fragments and to generate the different bacterial expression constructs.

Table 2.1: Primers and reaction conditions used to synthesize and subclone ADAMTS cDNA fragments.

<table>
<thead>
<tr>
<th>Primer Pairs*</th>
<th>PCR Type</th>
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<td>ca. 0.5 kb</td>
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<tr>
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<td>RT-PCR</td>
<td>ca. 0.4 kb</td>
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<tr>
<td>ATS-up2: AGCCCTGGTCCCVNTGYAS</td>
<td>RT-PCR</td>
<td></td>
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<td>ATS-low: AACTGACCTCCYSYNCCRCA</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>PCR</td>
<td>0.5 kb</td>
<td>15</td>
</tr>
<tr>
<td>ATS1-NotI-low: atgcggccgctcaaacacatggaggaatgactctcat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATS4-Sfil-up: ttggcttattgcacatggattctagtgttgttaacatcggagacat</td>
<td>PCR</td>
<td>0.37 kb</td>
<td>15</td>
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<tr>
<td>ATS4-NotI-low: atgcggccgctcaaacacatggaggaatgactctcat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATS5-Sfil-up: ttggcttattgcacatggattctagtgttgttaacatcggagacat</td>
<td>PCR</td>
<td>0.5 kb</td>
<td>15</td>
</tr>
<tr>
<td>ATS5-NotI-low: atgcggccgctcaaacacatggaggaatgactctcat</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

(*) N=A/C/G/T; R=A/G; S=C/G; V=A/C/G; Y=C/T. Bases complementary to ADAMTS sequences are written in capital letters. Blue marks the sequence encoding the factor-X cleavage site, red are restriction site sequences.

(RT-PCR): Degenerated RT-PCR using the OneStep RT-PCR Kit (1x OneStep RT-PCR Buffer, 400 µM dNTPs, 0.6 µM primers, 2 µl OneStep RT-PCR Enzyme mix in a total volume of 50 µl). Reverse transcription 50°C 30 min. Cycling conditions: 95°C 1 min, 50°C 1 min. Initial denaturation 95°C 15 min; final extension 72°C 10 min.

(PCR): Second round PCR using the Taq GOLD Polymerase (1x PCR buffer, 2.5 mM MgCl2, 0.2 µM of dNTPs, 0.2 µM primers, 2.5 U Polymerase in a total volume of 50 µl). Cycling conditions: 95°C 1 min, 68°C 1 min. Initial denaturation 95°C 10 min; final extension 72°C 10 min. Templates: cDNA fragments in pGemTeasy.

His-tagged fragments of ADAMTS proteases were expressed in E.Coli M15[pREP4]. The fusion proteins were isolated by chelating Ni²⁺-NTA agarose (Qiagen) columns and used for immunization of rabbits. Resulting antisera were purified by affinity chromatography employing a bacterial extract and corresponding fusion protein columns. For the detail
description of the preparation of recombinant fusion protein antigens and generation of polyclonal antibodies see Part I paragraphs 2.2.1. to 2.2.3.

2.2. Expression of Recombinant Full-length ADAMTS Endopeptidases

2.2.1. Generation of Mammalian Expression Vectors

Total RNA was prepared from mouse embryonic fibroblasts obtained from the trunk of E14.5 embryos. Primers were designed to anneal in the 5' and 3' untranslated regions of ADAMTS-1 (primer pairs: RT-ATS1-up/RT-ATS1-low), -4 (primer pairs: RT-ATS4-up/RT-ATS4-low) and -5 (primer pairs: RT-ATS5-up/RT-ATS5-low). The full-length cDNAs were amplified using the OneStep RT-PCR Kit (QIAGEN). PCR products were subcloned into pGemTeasy vector and completely sequenced. Point mutations generated during the PCR amplification were corrected by assembling non-mutated fragments from different subclones. For recombinant expression in mammalian cells, pSecTagA (Invitrogen) was chosen as vector. Proteins expressed from this vector are fused to the murine Ig κ-chain leader sequence for protein secretion and include at the C-terminus a peptide-tag containing the myc epitope and six consecutive histidine residues for detection and purification. To improve protein secretion two additional signal peptides were tested; the proper ADAMTSs sequence and the leader from the basement membrane protein BM-40.

A PCR amplification strategy was used to generate the constructs, pSecTag-PRO-ATSl, pSecTag-PRO-ATS4 and pSecTag-PRO-ATS5. In this vector variants the ADAMTS pro-peptides have been subcloned in frame with the Ig κ-chain leader sequence of the pSecTagA vector. Specifically modified primer sets were designed for each protease in order to incorporate a SfI restriction site at the 5' end of the gene and a XhoI site at the 3' end that facilitated the ligation of the PCR products in frame with the myc- at His-tag sequences. A similar strategy was used to subclone the entire coding region including the genuine secretory signal sequence of ADAMTS-1, -4 and -5 genes 5' prime to the myc and His tags. In this case the forward primers contained a Kozak sequence and a Nhel site at the 5' end of the gene, while the reverse primers were the same used to clone the pro-peptides. The PCR products were digested and ligated into the Nhel/XhoI cleaved pSecTagA vector, this way we obtained the pSecTag-SP-ATS1, pSecTag-SP-ATS4 and pSecTag-SP-ATS5 expression vectors.

All primer sequences and reaction conditions used to clone the full-length ADAMTS cDNAs and to generate the different mammalian expression vectors are summarized in Table 2.2.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Primer Pairs</th>
<th>PCR Type</th>
<th>Product Length</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGemTeasy-cDNA-ADAMTS1</td>
<td>RT-ATS1-up: CAGAAGCCTGCTGCCGACAT</td>
<td>RT-PCR</td>
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<td>25</td>
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<td></td>
<td>RT-ATS1-low: CCCCCCAGCCTACCTTG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pGemTeasy-cDNA-ADAMTS4</td>
<td>RT-ATS4-up: CGTTTGGTGCCGCCAGATGG</td>
<td>RT-PCR</td>
<td>2.6 kb</td>
<td>25</td>
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<tr>
<td></td>
<td>RT-ATS4-low: AGGCGACGGCGAGATGA</td>
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<td></td>
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</tr>
<tr>
<td>pGemTeasy-cDNA-ADAMTS-5</td>
<td>RT-ATS5-up: GCGGGCAGGCGACTATG</td>
<td>RT-PCR</td>
<td>2.9 kb</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>RT-ATS5-low: CTGGGATATCTCTGTTGGT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pSecTag-SP-ATS1</td>
<td>PRO-ATS1-up: cgcgtetagaACTGCACCTGTGTCATGGC</td>
<td>PCR</td>
<td>2.9 kb</td>
<td>15</td>
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<tr>
<td></td>
<td>PRO-ATS1-low: cgcgtetagaACTGCACCTGTGTCATGGC</td>
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<tr>
<td>pSecTag-SP-ATS4</td>
<td>PRO-ATS4-up: attagctagcaccATGTCCCAGATGGGCTTGC</td>
<td>PCR</td>
<td>2.5 kb</td>
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<td></td>
<td>PRO-ATS4-low: attagctagcaccATGTCCCAGATGGGCTTGC</td>
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<tr>
<td></td>
<td>PRO-ATS5-low: attagctagcaccATGCCTGCTAGATGGG</td>
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<td></td>
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</tr>
</tbody>
</table>

(*) Bases complementary to ADAMTS sequences are written in capital letters. Blue is the Kozak sequence, red are restriction site sequences.

(RT-PCR): RT-PCR using the OneStep RT-PCR Kit (1x OneStep RT-PCR Buffer, 400 μM dNTPs, 0.6 μM of primers, 1 μg total RNA template, 2 μl OneStep RT-PCR Enzyme mix in a total volume of 50 μl). Reverse transcription 50°C 30 min. Cycling conditions: 95°C 1 min, 68°C 4 min 30 s: Initial denaturation 95°C 15 min; final extension 72°C 10 min.

(PCR): Standard PCR using the Taq GOLD Polymerase (1x PCR buffer, 2.5 mM MgCl2, 0.2 μM dNTPs, 0.2 μM primers, 10 ng of plasmid template, 2.5 U Polymerase in a total volume of 50 μl). Cycling conditions: 95°C 1 min, 68°C 4 min 30 s. Initial denaturation 95°C 10 min; final extension 72°C 8 min.

The pSecTag-BM40 constructs were generated by exchanging the Ig κ-chain leader sequence in the pSecTag-Pro vectors with the leader sequence of the basal membrane 40 protein. For this purpose the primers BM40-UP (5’ ATT AGC TAG CCA CCA TGA GGG CCT G 3’) and BM40-LOW (5’ ATT AGG CCG GCT GGG CCG CCG CTG CCA GAG 3’) were designed to PCR amplify the BM40 signal peptide from the pCEP-ratNC vector (kind gift of Uwe Rauch). The 80 bp PCR product containing the NheI and SfiI restriction sites was then ligated into the corresponding sites of the pSecTag-PRO-ATS1, -ATS4 and -ATS5, giving rise to the pSecTagBM40-ATS1, -ATS4 and -ATS5, respectively.

All constructs were sequenced to verify that no spurious mutations were incorporated during PCR.

### 2.2.2. Transient and Stable Expression

COS-7 and HEK 293-FT (Invitrogen) cells were grown in D-MEM (Gibco, Life Technologies) supplemented with 10 % FCS (Winiger AG, Bio-Consulting), 1xGlutamax (Gibco, Life Technologies), 1 % penicillin/streptomycin (Gibco, Life Technologies) in a humified atmosphere at 37°C with 5 % CO2.
Cells were transiently or stable transfected either by electroporation or using the dendrimer based PolyFect transfection reagent (Qiagen). For electroporation, \(2 \times 10^6\) (COS-7) or \(5 \times 10^6\) (HEK 293-FT) exponentially growing cells were transfected with 20 or 50 \(\mu\)g of expression vector respectively. An electrical pulse of 0.24 kV, 750 \(\mu\)F was applied for a total volume of 800 \(\mu\)L. Transfection using the PolyFect reagent followed the protocol suggested by the supplier. Briefly, the day before transfection cells were seeded at about 40-60% confluence. 24 hours later a mixture of DNA and PolyFect transfection reagent was added to cells. To increase cell viability medium containing the PolyFect-DNA complex was replaced after 12 hours with normal growth medium.

In transient expression experiments the medium was replaced 2 days after transfection by serum-free medium containing 5 \(\mu\)g/ml heparin (SIGMA). Cells were grown for another 4 days before collecting the conditioned medium and cell fraction.

To generate cell lines stable expressing ADAMTS-1, -4 and -5, cells were subjected to Zeocine (Invitrogen) selection for 8 weeks.

### 2.2.3. Purification of His-Tagged ADAMTS

For protein purification using the Ni\(^{2+}\)-NTA agarose columns, serum-free conditioned medium was collected and clarified by centrifugation at 4000 rpm for 5 min at 4\(^\circ\) C. Purification was performed under native conditions. Briefly, medium supplemented with 50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, 10 mM imidazole, pH 8 was applied to a Ni\(^{2+}\)-NTA agarose column (Qiagen), after extensive washing with 50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, 20 mM imidazole, pH 8, recombinant proteins were eluted with 50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, 250 mM imidazole, pH 8. The eluate was then concentrated about ten fold using Microcon YM-10 filters (Millipore) and finally extensively dialysed against 50 \(\mu\)M Tris, 100 \(\mu\)M NaCl, 10 \(\mu\)M CaCl\(_2\), pH 7.5.

### 2.3. Electrophoresis and Immunoblotting

Protein samples were denatured in loading buffer (100 mM Tris-HCl, 10 mM EDTA, 20 % SDS, 3 % \(\beta\)-mercaptoethanol, 0.1 % bromphenol blue) for 8 min. at 95\(^\circ\) C. Following electrophoresis on PHAST polyacrylamide gels (Pharmacia, Uppsala, Sweden), proteins were detected by Coomassie Blue or Silver staining according to the supplier’s recommendations. For immunoblotting samples were run on PHAST polyacrylamide gels (Pharmacia, Uppsala, Sweden) and blotted onto Immobilon-P membranes (Millipore, Bedford, MA) by diffusion transfer at 70\(^\circ\) C for 30 min. After blocking in 3 % dry milk in TBS buffer for 30 min, membranes were incubated o/n with the first antibody (dilution 1:500-1:1000). After three washes in TBST buffer, binding of an alkaline phosphatase conjugated secondary antibody was allowed for 2 h at room temperature. Following three additional washes with TBST buffer, chromogenic detection was performed using the western blue substrate (Promega)
2.4. Enzymatic Assays

Recombinant bovine versican fragments expressed in bacteria were incubated with purified mouse ADAMTSs or with a commercially available human his-tagged ADAMTS-4 (Oncogene) at an appropriate ratio in 50 μM Tris, 100 μM NaCl, 10 μM CaCl₂, 1 μM leupeptin, 1 μM pepstatin, 1 mM peffablock pH 7.5 at 37°C for 16h. Following digestion samples were analyzed by immunoblotting as described above using primary antibodies against bovine versican N-terminal peptide (dilution 1:1000), GAG-α₁ domain (dilution 1:1000), or GAG-α₁₁ domain (dilution 1:1000) (Schmalfeldt et al., 1998).

For digestion of native substrate aliquots of versican preparations (kind gift of S. Raghav-Dutt) were incubated with ADAMTS proteases as described above. The samples were then deglycosylated by overnight digestion at 37°C with chondroitinase ABC (Seigaku, Japan). Finally digests were run on 4-12 % PHAST gels (Pharmacia) for immunoblot analysis.

2.5. RT-PCR

For the analysis of ADAMTSs expression by RT-PCR, total RNA was extracted from mouse embryos at different developmental stages or from different organs of adult animals using the RNeasy Maxi, Midi or mini kits (Qiagen). RT-PCR was performed using the OneStep RT-PCR kit (Qiagen); primers were chosen to hybridize to the catalytic region and the first thrombospondin motif of the different proteases. Since the amplified regions span over more than one exon, amplification from contaminating genomic DNA was avoided. Amplification of the housekeeping gene GAPDH was used as internal control Table 2.3 summarizes all primer sequences and reaction condition used, additional information about primer positions are given in the results section.

Table 2.3: Primer sequences and reaction conditions used in RT-PCR experiments

<table>
<thead>
<tr>
<th>mRNA Detected</th>
<th>Primers Pairs*</th>
<th>Product length</th>
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<td>499 bp</td>
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<td>low: atgcggccgctcaAAACAGGAGTAGC</td>
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<td>ADAMTS-5 up: tggccctttattggccattgaagtcgtCTACTG</td>
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<td>low: atgcggccgctcaCCAATTTCACCATGGCTAGTC</td>
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<tr>
<td>GAPDH up: ACCGACCCCTTCTG</td>
<td>191 bp</td>
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<td></td>
</tr>
<tr>
<td>low: TCCACGACATTACTCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*): Bases complementary to ADAMTS sequences are written in capital letters.

RT-PCR using the OneStep RT-PCR Kit (1x OneStep RT-PCR Buffer, 400 μM dNTPs, 0.6 μM primer, 1 μg total RNA template, 2 μl OneStep RT-PCR Enzyme mix in a total volume of 50 μl). Reverse transcription 50°C 30 min. Cycling conditions: 95°C 1 min, 65°C 3 min 30 s Initial denaturation 95°C 15 min; final extension 72°C 10 min.
2.6. Neurite Outgrowth Assay

2.6.1. Preparation of the Stripe Coated Coverslips

Glass coverslips (Menzelgläser, 18x18 mm) were first boiled in 65 % nitric acid for 5 minutes, extensively washed in ddH2O and incubated at 50° C overnight. The dried coverslips were then incubated for 30 min. in 0.125 mg/ml poly-L-lysine (Sigma) dissolved in ddH2O, shortly washed in ddH2O and dried at 50° C overnight. Nitrocellulose coating was performed by pipetting 100 µl nitrocellulose BA85 (0.85 cm2/ml in methanol, Schleicher and Schüll, Germany) onto the surface of the coverslips. The solution was allowed to air dry at room temperature for about 30 min. They were then again dried at 50° C overnight, and finally stored until use in a dessicator containing silica crystals.

To generate a pattern consisting of stripes of test and control substrates on the surface-treated coverslips, silicon matrices were used (Max-Planck-Institüt für Entwicklungsbiologie, Tübingen, Germany) (Figure 2.3). These matrices were applied onto the coverslips. Than 25 µl of the test substrate mixed with laminin-1 (final concentration 100 µg/ml), were injected into the channel system. The substrate was allowed to bind to the nitrocellulose-coated surface for 1 h, the channels were than extensively rinsed with Hank’s buffer (GIBCO, Life science) in order to wash away unbound proteins. The silicon matrix was than removed and the coverslip surface further coated with 20 µg/ml laminin-1 for 1 h. The processed coverslips were finally blocked for 1 h with DMEM/F12 medium (Gibco, Life Technologies) supplemented with 10 % FCS (Winiger AG, Bio-Consulting), 2 % chicken serum (Gibco, Life Technologies), 100 U/ml penicillin (Gibco, Life Technologies), 100 µg/ml streptomycin (Gibco, Life Technologies), 2 mM L-glutamine (Gibco, Life Technologies) and 0.5 % methylcellulose (Sigma).

![Figure 2.3: Silicon matrix used for coating of test substrates onto glass coverslip](image-url)
2.6.2. Preparation of Chicken Retinal Ganglion Explants

Retinal ganglion cell (RGC) explants were prepared from E7 chick embryos (stage 31-32). The embryos were decapitated, the eyeballs were excised and transferred in 10 cm dishes containing Hank’s buffer. The eyes were further dissected under a stereomicroscope in order to obtain the retinal layer cleared of all the connective tissue, cornea, lens, pigment epithelium, and the vitreous body. The retina was than flat-mounted onto a 0.45 µm black nitrocellulose filter (Sartorius AG, Germany) previously treated with 0.1 mg/ml Con A (Sigma). About 0.5 mm wide explant stripes were than cut with a scalpel and transferred to the previously coated coverslips. The explants were placed perpendicular to the coated stripes with the cell layer facing down. Small weights were applied to stabilize the explants and to avoid floating of the coverslips. The cultures were allowed to proceed for about 2 days in medium (DMEM/F12 medium, 10 % FCS, 2 % chicken serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.5 % methylcellulose), prior to processing for immunofluorescence staining.

For immunohistological stainings, the cultures were fixed with 4 % paraformaldehyde/0.33 % sucrose in PBS for 10 min at RT. Following blocking in 0.5 % BSA/0.2 % gelatine in PBS/NaN₃ for 2 h at RT, the cultures were incubated with anti-versican polyclonal antibodies recognizing the N-terminus and the GAGα-domain (GAG-αI and GAG-αII, each diluted 1:100 in blocking buffer). Incubation with the primary antibodies was performed over night at RT in a humid chamber. The cultures were washed twice for 15 min at RT with PBS to remove unbound antibodies, followed by 2 h incubation at RT with Alexa-labelled fluorescent secondary antibodies (anti rabbit IgG Alexa-488, diluted 1:200). After three washes with PBS, cultures were embedded in DakoCytomation fluorescent mounting medium for storage.
3. Results

3.1. Generation and Characterization of Polyclonal Antibodies Against ADAMTS Proteases

cDNA fragments of different ADAMTS proteases were obtained by RT-PCR using degenerated primers annealing to regions conserved between different ADAMTS family members. All known ADAMTS cDNA sequences from various species (mouse, rat, human, and bovine) were compiled and aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw). That way we could find short conserved regions located in the catalytic domain and in the first thrombospondin motif of the proteases. In these regions we designed degenerated primers pairs that we used in RT-PCR experiments.

As template for the RT-PCRs we used total RNA extracted from entire E14 mouse embryos and adult mouse brains. cDNA fragments showing the expected size were subcloned the into pGemTeasy vector and sequenced. That way we obtained from total RNA of E14 mouse embryos cDNA fragments of ADAMTS-1, -5 and -7 whereas from adult mouse brain total RNA we amplified fragments of ADAMTS-1, -4, and -5. The ADAMTS-1, -4, -5 amplificates were further processed to prepare bacterial fusion proteins that after purification were used for the immunization of rabbits.

The purified polyclonal antibodies were tested on fusion protein immunoblots for their reactivity. All antibodies recognized the corresponding fusion proteins, furthermore since poorly conserved regions were chose as antigen, the antisera displayed no cross-reactivity among the different ADAMTS family members.

![Figure 3.1] (A) Schematic representation of the ADAMTS-1 gene and mRNA showing the annealing site of the degenerated primers (orange arrowheads). (B) The corresponding RT-PCR products (yellow arrowheads) were cloned and sequenced. This way we obtained cDNA fragments of ADAMTS-1, -4, -5, and -7. (C) Antibodies against His-tagged ADAMTS-1, -4 and -5 fusion proteins show no cross-reactivity in immunoblots.
3.2. ADAMTS-1, -4, and -5 Expression During Embryonic Development and in Adult Mouse Tissues

The expression pattern of ADAMTS-1, -4, and -5 was examined in a variety of adult and fetal mouse tissues employing RT-PCR. Since a one-step RT-PCR approach was used, primers were chosen to anneal at distinct exons thus avoiding amplification from contaminating genomic DNA. As control for template integrity and RT-PCR procedure, primers for the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) housekeeping gene were added to each reaction. Signals for ADAMTS-1, -4, and -5 were observed at all the embryonic stages analyzed (Figure 3.3A). Similarly transcripts for ADAMTS-1 and -5 were detected in all adult organs analyzed, whereas ADAMTS-4 appears to be specifically expressed in the brain (Figure 3.3B).

![RT-PCR Analysis](image)

**Figure 3.2:** RT-PCR analysis of mouse ADAMTS-1, -4, and -5. (A) RT-PCR using total RNA extracted from mouse embryos at different stages. (B) Analysis of total RNA extracted from different adult organs. The expected product size for the ADAMTS-1, -4, -5 primers are 499 bp, 376 bp, and 508 bp, respectively. As internal control primers for the GAPDH were used (expected product size is 191 bp).

3.3. Recombinant Expression of ADAMTS-1, -4, and -5

The full-length cDNA of ADAMTS-1, -4 and -5 were obtained by RT-PCR using primers annealing to the 5'- and 3'-untranslated region of the corresponding mRNAs. As template we used total RNA extracted from mouse embryonic fibroblasts obtained from E14 embryos. This way we obtained 2.9, 2.6, and 2.9 kb long cDNA fragments corresponding to the coding sequences of ADAMTS-1, -4 and -5, respectively. The sequences were subcloned into a pSecTag vector for expression in mammalian cells.
Figure 3.3: (A) cDNA products obtained by RT-PCR corresponding to the full-length coding sequences of mouse ADAMTS-1, -4, -5. (B) These sequences were subcloned into the pSecTag vector, in which expression is driven by the strong CMV promoter. The expression product is fused to a myc epitope and six consecutive histidine residues for protein detection and purification. To improve protein secretion, we exchanged the Ig κ-chain leader sequence contained in the original expression vector with the BM-40 signal sequence.

In our initial attempt to produce recombinant ADAMTSs we transiently transfected COS-7 cells. Expression of the recombinant proteases could be detected by western blotting in cell extracts of transiently transfected cells. Using antibodies against the C-terminal myc epitope, the his-tag epitope (data not shown) or using our antibodies raised against an internal portion of the proteases, each ADAMTS-1, -4, and -5 appeared as two distinct bands. Based on the calculated molecular weights these bands probably represent the zymogen and the activated forms of the three ADAMTS endopeptidases.

These results prove not only that our vectors are suitable to produce recombinant ADAMTS but also that COS-7 cells posses the enzyme needed for zymogen activation. However, we could detect no or only low levels of recombinant proteins in the supernatant fractions and attempt to purify them from the conditioned medium failed.

Figure 3.4: Transient expression of ADAMTSs in COS-7 cells. Our antibodies as well as the anti myc antibodies recognized the zymogen (arrow) and the activated form (arrowhead) of the three proteases in crude cell pellet extracts (lane 1). No signals were detected in the conditioned medium (lane 2) of transiently transfected cells. The calculated molecular weight are: ADAMTS-1: zymogen = 105,731 kDa, activated form = 81,458 kDa; ADAMTS-4: zymogen = 89,973 kDa, activated form = 70,605 kDa; ADAMTS-5: zymogen = 104,683 kDa, activated form = 76,689 kDa.
Since other groups reported efficient recombinant expression of ADAMTSs in human 293 cells, we decided to use a derivative of this cell line (293FT cell line, Invitrogen) to generate clones that stably express ADAMTS-1, -4, or -5. Stable transfected cells were subjected to two month of antibiotic selection then single clone were sorted and tested for ADAMTSs expression.

Western blot analyses revealed efficient secretion of both ADAMTS-1, and -4, whereas no bands could be seen in the conditioned medium of cells expressing ADAMTS-5. Furthermore using our antibodies, we could detect the occurrence of C-terminal proteolytic events for both ADAMTS-1, and -4, resulting in several bands smaller than those for the zymogen or the activated form of the enzymes after propeptide cleavage. Since C-terminal processing resulted in the loss of the His-tag we were unable to purify ADAMTSs from the conditioned medium of stable transfected cells.

Since in the meantime recombinant human ADAMTS-4 corresponding to a C-truncated form became commercially available we performed the subsequent in vitro experiments with these preparations.

![Figure 3.5: Stable expression of ADAMTSs in 293 FT cells. Our antibodies detected the presence of C-terminal truncated forms of ADAMTS-1, and -4 (red arrowhead) lacking the C-terminal his-tag needed for purification.](image)

### 3.4. Digestion of Versican V2 by h.ADAMTS-4 Reduce its Activity in Inhibiting the Outgrowth of Neurites in Stripe Choice Assays

First we analyzed the versicanase activity of the recombinant human ADAMTS-4. For this purpose we performed several digestions using our previously purified bovine versican V2 as substrate. Following incubation with ADAMTS-4 the versican samples were subjected to chondroitinase ABC processing to remove the GAG-chains and analyzed by Western blotting with a set of antibodies raised against three different regions of the versican V2 core protein (Figure 3.7).

The digestion of versican with this ADAMTS-4 preparation gave rise to a number of degradation products, the process was however, never complete. Consequently, some of the full-length substrate remained as a single band of about 400 kDa on the top of the gel. Three additional bands of approximately 85, 55, and 30 kDa were detected using the N-terminal antibody. The two bands of 85 and 55 kDa bands were also recognized by the α1 antibody, which bound to additional bands with molecular weight higher than 200 kDa. The 55 kDa band probably represent the previously characterized GHAP (glial hyaluronate-binding protein) versican V2 cleavage product, whereas the identity of the other fragments is
unknown, the band of about 90 kDa recognized by both the N-terminal and αI antibodies may be an artifact which is seen only with the PHAST gels, similar artifacts are also observed by other members of the lab using this system. The αII antibody, was not reactive against the 85, 55, and 30 kDa fragments but recognized several high molecular weight bands, which may correspond to the C-terminal counterparts of these fragments.

Figure 3.6: (A) Analysis of the versican core protein fragments generated after ADAMTS-4 cleavage (lane 2, 3, and 4). Digested versican V2 was analyzed using a N-terminal antibody (lane 2), the α1 antibody (lane 3), and the αII antibody (lane 4). Undigested versican was visualized using the N-terminal antibody (lane 1) M: molecular weight marker. (B) Localization of the known ADAMTS cleavage site (red arrow) in the versican V2 core protein. Putative positions of additional cleavage sites are indicated with green arrows. All samples were chondroitinase ABC treated.

We previously showed that the brain specific versican V2 isoform inhibits the outgrowth of neurites from dorsal root ganglion (DRG) and retinal ganglion cells (RGC) in a concentration dependent manner (Schmalfeldt et al., 2000). In our in vitro stripe choice assays, neuronal explants were plated on glass coverslips adsorbed with a patterned substratum consisting of alternating stripes of laminin (LN) or versican V2 plus laminin. A versican V2 concentration of 50 pg/ml was already sufficient to restrict the growth of neurites to the laminin alone lanes. To test whether ADAMTS-4 mediated versican digestion had an effect on its inhibitory activity on neurite outgrowth, we tested full-length and cleaved versican V2 on stripe choice assays using chick RGCs. As expected at concentrations of 50 μg/ml, neurites avoided the lanes containing full-length versican V2 mixed with laminin. The inhibition of neurites extension by versican V2 was partly reduced by ADAMTS-4 digestion, in fact at the same concentration the neurites displayed an increased tendency to cross the versican containing lanes. A less pronounced reduction of the versican inhibitory activity was also observed by removal of the GAG chains by digestion with chondroitinase ABC. The inhibitory effect of versican was strongly reduced, but not completely abolished when versican was digested with both enzymes. In the control experiments, using 100 μg/ml laminin-1 or ADAMTS-4 plus laminin-1 applied in stripes alternating with 20 μg/ml laminin-1, a random neurite outgrowth pattern was observed.
Figure 3.7: Effect of ADAMTS-4 and chondroitinase digestion on the neurite outgrowth inhibiting activity of versican V2. The neurites from E7 chick retinal explants avoid stripes coated with versican plus laminin-1 (green), but extend along the lanes that contain laminin-1 alone. A partial reduction of the inhibitory activity was observed upon digestion of versican V2 with h.ADAMTS-4 or chondroitinase ABC, whereas maximal effects were observed when both enzymes were used. Coating solution included 50 μg/ml undigested or digested versican V2 plus 100 μg/ml laminin-1 and 20 μg/ml laminin-1.
4. Discussion

4.1. Expression Pattern Analyses of ADAMTS

The rapid turnover of versicans in tissues that form transitional barriers to the ingrowth of peripheral axons during embryonic development has prompted us to study the tissue distribution of hyalectan degrading ADAMTS proteases (Figure 4.1). For this purpose, we have generated polyclonal antibodies against specific portions of these enzymes.

Figure 4.1: Versican V0/V1 expression in the developing chicken limb bud. The rapid disappearance of versican, at time of limb bud innervation, suggests the presence of a versican degrading activity. Arrowhead: extending axon; pg: pelvic girdle precursor cells (Landolt et al., 1995).

To date only ADAMTS-1, -4 and -9 have been reported to cleave versican in in vitro assays. However, only a few ADAMTS proteases have been studied extensively for their substrate specificity leading to the possibility that other family members may be involved in the versican turnover. At the beginning of this project only the mouse sequences encoding for ADAMTS-4 and -8 had been determined. For these reasons we decided to perform RT-PCRs with degenerated primers annealing at regions conserved between the different ADAMTSs, using as template total RNA from tissues known to express versican (whole mouse embryos and adult brain). That way we expected to obtain cDNA fragments of distinct ADAMTSs that potentially would colocalize with versican. Indeed we could obtain cDNA fragments of ADAMTS-1, -4, and -5, which are known to digest hyalectans in vitro.

The antibodies raised against these three proteases showed strong reactivity against the corresponding bacterial fusion as well as the full-length recombinant proteases. Furthermore no cross-reactivity among the different ADAMTS family members could be detected, demonstrating the specificity of the single antibodies. However, the use of our antibodies for immunohistochemistry has so far been unsuccessful. Staining of mouse embryonic sections, with the purified antibodies yielded no specific signal, whereas the use of unpurified reactive antisera gave a strong background. Failure to detect the proteases could be due to the use of formalin fixed and paraffin embedded tissues, which may destroy the epitopes recognized by
the antibodies. Furthermore, is it likely that ADAMTSs, being active enzymes, might be expressed only at very low levels making their immunohistochemical detection very difficult. Our RT-PCR analyses indirectly suggest an involvement of ADAMTS-1, -4, and -5 in the rapid degradation of versican V0/V1 occurring during the late embryonic early postnatal period. Signals for all three proteases were also detected in the adult brain, indicating that they may be involved in the natural turnover of versican V2. Of particular interest in this context is ADAMTS-4, which in the adult organism seems to be specifically expressed in the brain. Recently ADAMTS-4 has been proposed as prime responsible for the cleavage of versican V2 (Westling et al., 2004) and brevican (Matthews et al., 2000) in the central nervous system.

In conclusion, our RT-PCR analysis suggests a potential co-localization of ADAMTSs with versicans, however the detection of the corresponding transcripts does not finally prove that the proteases are indeed active in versican degradation in vivo. Additional analysis by in situ hybridization and/or immunohistochemistry may provide more information about the precise localization of ADAMTSs and may further support the evidence of their direct involvement in the catabolism of versicans.

4.2. Digestion of Bovine Versican V2 by Human ADAMTS-4

Members of the ADAMTS family are known to cleave versicans, and other hyalectans, in vitro (Tang, 2001; Apte, 2004). Furthermore versican fragments generated after cleavage correspond to degradation products found in crude protein extracts from aorta and brain as demonstrated with antibodies raised against the putative neoepitopes resulting from ADAMTS cleavage. However the biological significance of this proteolysis is still unclear.

In the initial phase of this project we tried to express and purify three different mouse ADAMTSs with the intention to analyze their versicanase activity in in vitro experiments. Despite conditioned medium of transfected cells showed protease activity against recombinant versican fragments prepared in bacterial system (data not shown), we were unable to purify ADAMTS-1, -4, -5. This was mainly due to C-terminal processing of ADAMTSs leading to removal of the his tag needed for purification.

Thus we used, for our in vitro experiments, a commercially available human ADAMTS-4 corresponding to a p40 C-terminally truncated form of the enzyme (amino acids: 213 to 579 of human ADAMTS4 with a C-terminal His-tag). This enzyme actively cleaved our versican V2 preparation previously purified from bovine spinal cord. However incubation of bovine versican V2 with human ADAMTS-4 usually resulted in an incomplete digestion of the versican core protein. Kashiwagi M. et al. reported that the p70 polypeptide of the human ADAMTS-4 (amino acids 213 to 837) or a p53 C-terminally truncated form (amino acids 213 to 687) are four to five times more efficient in degrading aggrecan than a p40 fragment (amino acids 213 to 575) (Kashiwagi et al., 2003). This suggests that C-processed forms of ADAMTS-4 other than the commercially available p40 fragment used in our experiments will be more suitable to obtain a complete digestion of native versican V2.

Interestingly, our results suggest that the core protein of the bovine versican V2 contains additional ADAMTS cleavage sites other than the previously characterized site Glu410-Gln411 residing in the N-terminal portion of the GAG-α domain (Glu405-Gln406 for the human versican V2 sequence (Westling et al., 2004). In particular, the staining with the N-terminal αI antibodies suggest the presence of at least two additional cleavage sites, the first probably located within the N-terminal domain and the second close to the C-terminal end of the region.
recognized by the αI antibody or in the sequence between the two antibody-binding core protein portions (Figure 3.6).

4.3. Members of the ADAMTS Family Appear to Modulate Versican Activity

In this report we demonstrated that the in vitro inhibitory activity of versican V2 on the outgrowth of neurites from chicken retinal explants, can be partly reverted by ADAMTS-4 mediated cleavage. Since incubation of versican V2 with the commercially available recombinant ADAMTS-4 fragment (p40) usually resulted in an incomplete digestion, it is tempting to assume that a complete digestion with the more active p53 fragment of ADAMTS-4 might lead to an even more pronounced neutralizing effect. As already observed in our previous studies removal of the chondroitin sulfate chains alone had only a minor effect on the inhibitory activity of versican, while maximal but still not complete reversal of the inhibition was obtained by combined treatment with ADAMTS-4 and chondroitinase ABC.

Little is known about the mechanisms by which versican inhibits neurite outgrowth. However evidence is accumulating that interactions with cell surface adhesion molecules and direct growth cone signaling are important (Sandvig et al., 2004). Hyaluronan, one of the versican ligands, has been found to accumulate on various cell surfaces probably by interaction with its potential receptors (CD44, RHAMM, LYVE-1, and Toll-like receptor 4) or by staying attached to the membrane-associated hyaluronan synthases. One can hypothesize that incorporation of the large and highly hydrated versican into this well organized hyaluronan network likely to cover also the growth cone may sterically hinder the interaction of integrins and/or other cell surface receptors with adhesion and/or growth-promoting cues present in the extracellular space. In such a model mechanism, ADAMTSs activity could induce the release of the C-terminal versican portion containing the GAG chains and thus may revert the sterical effect.

A second model proposes the existence of a currently unknown versican signaling receptor on the growth cone. Such a receptor complex has been recently identified for the three majors CNS myelin-associated inhibitors, Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), the myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and the oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002). All of them exert their inhibitory activity through a common receptor complex consisting of the GPI-anchored Nogo-66 receptor (NgR), and the neurotrophin receptor p75NTR. The p75NTR/NgR receptor complex transduces the inhibitory signal by activating the Rho/ROCK pathway, which finally signals to the actin cytoskeleton leading to the rapid collapse of the growth cone.

There is increasing evidence that also CSPGs signal through the Rho/ROCK pathway. It seems however, that the modulation of RhoA and Rac activity is at least in the case of versican V2 independent from the p75NTR/NgR receptor complex (Schweigreiter et al., 2004). The hypothesis that versican may, directly or indirectly, modulate the activity of a to date unknown cell surface receptor, which will transduce the inhibitory signal across the membrane to the neuron is further supported by recent results from time lapse videomicroscopy demonstrating that versicans does not only passively inhibit neurite growth but actively induces growth cone collapse in vitro (Niederost et al., 1999) (S. Dutt-Raghav manuscript in preparation). In this model, the degradation by ADAMTS proteases during development could rapidly remove the ligand of this putative versican-receptor at the beginning of the signaling cascade.
4.4. Functional Implications of ADAMTSs Mediated Cleavage of Hyalectans

Proteolytic remodeling of the ECM has been implicated in a number of important biological and pathological processes. The physiological role of hyalectan degrading ADAMTS proteases in the CNS has not yet been elucidated. Our in vitro results suggest that ADAMTS might modulate the inhibitory activity of hyalectans on axonal outgrowth. If this occurs also in vivo it might be a refined mechanism to modulate axonal navigation during nervous system development being able to rapidly convert a repulsive extracellular matrix barrier to a growth promoting pathway. Similarly, hyalectan degradation might enhance also cell migration on otherwise non-permissive ECM substrates during normal neurogenesis and in pathological processes such as tumor progression. Interestingly production and proteolytic processing of brevican by ADAMTS-4 have been associated with increased infiltrative activity of gliomas (Matthews et al., 2000).

There are indications that hyalectans may function to limit plasticity and prevent the formation of aberrant connections in the intact adult CNS (Yamaguchi, 2000; Rauch, 2004). In fact aggrecan, neurocan and brevican have been found in perineuronal nets (PNN) (Koppe et al., 1997; Koppel et al., 1997; Bruckner et al., 1998; Matsui et al., 1998; Matthews et al., 2002), a specialized extracellular matrix present on the surface of neuronal cell bodies and proximal dendrites. Since neuronal surfaces covered by PNNs are devoid of synaptic contacts (Bruckner et al., 1993), it has been proposed that PNNs may specifically serve to block formation of new synapses (Celio and Blumcke, 1994). Recently, Yamaguchi proposed a model of PNNs whereby long hyaluronan molecules bind hyalectans (lecticans), which in turn bind tenascin and form net-like complexes between neurons and glia (Figure 4.2) (Yamaguchi, 2000). Due to the axon growth inhibitory property of both, hyalectans and tenascins, this net-like complex may act as a highly repulsive barrier against approaching axons and dendrites. ADAMTS mediated hyalectan cleavage will result in the disruption or weakening of this meshwork structure, making the environment more permissive for the establishment of new synaptic contacts. Following this idea, one could for instance speculate that the expression and activation of ADAMTS endopeptidases in the adult hippocampus is required to promote synaptic plasticity and therefore may be actively involved in learning and memory processes.

Figure 4.2: The HLT (hyaluronan-lectican-tenascin) model suggested by Yamaguchi: Lecticans (hyalectans) form complexes with hyaluronan and tenascins in the brain ECM and in the perineuronal nets (Yamaguchi, 2000) whereby stabilizing synaptic connections and reducing plasticity.
Complexes of hyaluronan, hyalectans and tenascins may also suppress regenerative processes in the adult brain and spinal cord. Following injury to the CNS, the expression of a number of ECM molecules increases in regions of reactive gliosis. The glial scar includes various CSPGs, some of them belonging to the hyalectan family, which have been implicated in the inhibition of axonal growth. Furthermore, experimental treatment with chondroitinase ABC resulted in limited fiber regeneration in in vivo- and in vitro-models of CNS injury (Zuo et al., 1998b; Yick et al., 2000; Krekoski et al., 2001; Moon et al., 2001; Bradbury et al., 2002; Zuo et al., 2002; Yick et al., 2003). Since chondroitinase ABC is a glycosidase that digests the chondroitin sulfate GAG side chains of proteoglycans, the increased regeneration capacity was attributed to the degradation of the carbohydrate moiety of the CSPGs. However, the core proteins of CSPGs also possess intrinsic inhibitory properties suggesting that additional degradation of the polypeptide backbone by specific proteases might enhance the neutralizing effect. Similarly, Zuo et al. reported that axonal growth by DRG cultured on normal peripheral nerves sections was markedly increased by first treating the nerve sections with MMP-2 (Zuo et al., 1998a). Nevertheless, MMPs might not be suitable for clinical applications, as they display a broad substrate specificity, which may make it difficult to control potential side effects. In contrast, several ADAMTSs seem to specifically cleave the axon growth inhibitors of the hyalectan family. Thus, ADAMTS may be more appropriate candidates for a selective therapeutic approach to treat patients after CNS injuries in the far future.
5. Outlook

Despite recent reports that members of the ADAMTS family degrade versicans in vitro, it is still unclear whether ADAMTSs are indeed responsible for the in vivo turnover of the different isoforms. Additional immunohistochemical and in situ hybridization studies showing a tight correlation of ADAMTS expression with versican removal will be required to answer this question. Our preliminary experiments suggest that the technical problems that we have encountered in the first immunohistochemical stainings of paraffin sections with our antibodies against ADAMTSs, can be overcome by changing to cryosections. Furthermore, the set of developmental time points analyzed in this initial study can be improved. Since our recent observations indicated a late start of versican V0/V1 degradation during CNS development, we would like to extend the immunohistochemical analyses of versicans and ADAMTSs expression to also include early postnatal stages. This should allow us to identify the period of maximal versican V0/V1 degradation and increase the chance to detect ADAMTS expression.

Using the stripe choice assay, we have shown that ADAMTS-4 modulates the inhibitory activity of versican V2 on the outgrowth of neurites in vitro. Earlier immunohistochemical studies in our group suggested that the large isoforms V0 and/or V1 play a more direct role in axon guidance during development, whereas versican V2 might be mainly responsible for the stabilization of the adult neuronal network in the CNS. We would therefore like to include also ADAMTS-degraded V0 and V1 splice-variants in our functional studies in vitro. Since a complete digestion of purified versican V2 with the commercial ADAMTS-4 preparation could neither be achieved by increasing the enzyme concentration nor by extending the incubation time, we plan to produce our own enzyme stocks for these experiments. We will do that by expressing different C-terminally truncated recombinant fragments of mouse ADAMTS-1, -4, and -5, and by selecting the enzyme form with the highest versicanase activity. These enzymes will be used to complete our in vitro functional studies with purified versican V0/V1, V1 and V2 substrates.

In a next phase we plan to extend our experiments on the ADAMTSs activity to a more complex in vivo situation. In previous work we demonstrated that the innervation of the limb bud in chicken correlates with a rapid disappearance of versican V0/V1 (Figure 4.1) (Landolt et al., 1995). Recently we could demonstrate that ectopical injections of versican in chicken limb buds in ovo interfere with the normal migration, fasciculation and branching of developing fiber tracts in the leg. This supports the hypothesis that versicans plays indeed an active role in guiding axons in the periphery (S. Raghav-Dutt, manuscript in preparation). In an analogous project, we would like now to analyze, whether the innervation is also disturbed by a premature degradation of versican V0/V1. Alternatively, we will try to delay the versican degradation by inhibiting ADAMTSs expression using RNA interference (RNAi) techniques. For this purpose we plan to inject active ADAMTS proteases in the chicken limb bud at a time point, when versican is still present at high concentrations, hoping to see a precocious immigration of the axons into the early limb anlagen and we will try to introduce double stranded RNA fragments in an in ovo electroporation approach.
References


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