Galactosaminoglycans - Role in Brittlestar Limb Regeneration

RASHMI RAMACHANDRA

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Abstract

Regeneration is, in simple terms, ‘to re-grow’ damaged or lost parts of the body (e.g. cells, tissues and organs) and is a natural phenomenon occurring throughout the life of an organism. The regenerative capacity varies in the animal kingdom. Invertebrates have high regenerative capacity in contrast to higher vertebrates. This raises several fundamental questions related to the regeneration potential, evolutionary selection and its cellular and molecular mechanisms. An in-depth knowledge in regeneration is warranted to answer the fundamental questions that are still a challenge in regenerative medicine.

Glycosaminoglycans (GAGs) are known to be involved in various physiological processes. Of several GAG types galactosaminoglycans are the focus of this thesis. Galactosaminoglycans such as chondroitin sulfate/dermatan sulfate (CS/DS) are anionic linear polysaccharides covalently linked to core proteins so called proteoglycans (PGs), and form an integral part of both cell surface and extracellular matrix components. Although CS/DS have been associated with different cellular processes from development to homeostasis, not many studies have been carried out to understand their role in regeneration. In this thesis, we aim to study galactosaminoglycans, their structure, and interaction with growth factors of biological importance in the process of regeneration using simple invertebrate model organisms - brittlestars.

We have identified CS/DS as the major GAG present in brittlestars. Molecular characterization of these chains indicated a much higher level of sulfation in Amphiura filiformis than so far found in GAGs from invertebrates or vertebrates. This brittlestar CS/DS promotes FGF2 mediated cell signaling similar to heparin. Further, we studied the functional role of these CS/DS chains and their biosynthetic machinery during arm regeneration in A. filiformis. Regeneration is followed by an increase in GAG sulfation from blastema stage to the fully functional arm. Suppressing sulfation on the other hand by sodium chlorate treatment drastically affected the proliferation process and thereby regeneration. Thus our findings suggest a potential biological role of CS/DS in brittlestar limb regeneration that may have relevance to regenerative medicine in future.

Keywords: Chondroitin sulfate, Dermatan sulfate, Brittlestar, Regeneration

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To my family and teachers
Salutation to the noble Guru, beyond whom there is no higher truth, there is no higher penance and there is nothing higher attainable than the true knowledge.
This thesis is based on the following manuscripts, which are referred to in the text by their Roman numerals.


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<tbody>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>C4ST</td>
<td>Chondroitin 4-O-sulfotransferase</td>
</tr>
<tr>
<td>C6ST</td>
<td>Chondroitin 6-O-sulfotransferase</td>
</tr>
<tr>
<td>ChPF</td>
<td>Chondroitin polymerizing factor</td>
</tr>
<tr>
<td>ChSy</td>
<td>Chondroitin synthase</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
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<td>Chondroitin sulfate proteoglycan</td>
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<td>Dermatan 2-O-sulfotransferase</td>
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<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1 and 2</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
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<td>Fibroblast growth factor receptor</td>
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<td>Galactosyl transferase</td>
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<tr>
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<tr>
<td>IdoA</td>
<td>L-Iduronic acid</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>OST</td>
<td>O-sulfotransferase</td>
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<tr>
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<td>3’-phosphoadenosine 5’-phosphosulfate</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>RPIP-HPLC</td>
<td>Reverse-phase ion pairing-high performance liquid chromatography</td>
</tr>
<tr>
<td>U2OST</td>
<td>Uronyl 2-O-sulfotransferase</td>
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<tr>
<td>UDP</td>
<td>Uridine-5’-diphosphate</td>
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Introduction

Regeneration

All organisms have evolved strategies to maintain their structure and form, as well as to deal with injuries and damage caused throughout their adult life mainly by a process of replacement called regeneration. The term regeneration is, in a broad sense ‘to grow again’. Replacement of cells (e.g. vertebrate blood cells, skin epithelium), wound healing (e.g. epithelial wound), regrowth of lost organs (e.g. limb, eye, tail in amphibians) or regrow the whole animal (e.g. annelida) from damaged part are all examples of regeneration.

Regeneration varies significantly with the stage in the life cycle (embryonic, larval or adult) and even the age of the individual. Considering the association between regeneration and cellular pluripotency different strategies have been developed during metazoan evolution to regulate it. However, there are obvious and important differences in regeneration potencies between lower and higher metazoans.

Higher animals begin their life as a single pluripotent cell and embryos have full capacity of regeneration that is eventually lost as development proceeds. This is due to tight regulation of pluripotent cells by various checkpoints at different stages of the life cycle [1]. Mammalian models to study cellular and molecular aspects of adult regeneration are restricted only to a few species e.g. Urodele amphibians (Newts, salamanders and frogs) [2] and Zebrafish [3] due to their cellular complexity and very limited regeneration potential confined to certain tissues and organs. Lower invertebrates are good model systems due to their simple tissue structure and high regenerative capacity. Studies on fundamental cellular and molecular mechanism in such organisms may provide cues on regeneration in higher vertebrates like mammals.

“If there were no regeneration there could be no life and if everything regenerated there would be no death” [4]. All organisms exist between these two extremes. This raises several fundamental questions related to the regeneration potential, its diversity in different organisms, evolutionary selection of this phenomenon and the cellular and molecular mechanisms active during the process. An in-depth knowledge is warranted to understand such a
complex process implicating potential therapeutic strategies in regenerative medicine.

**Cellular and molecular aspects of regeneration**

Regeneration involves two alternative processes based on absence or presence of cell proliferation:

a) *Morphallaxis* - a phenomenon of recreation of an individual by rearrangement/recycling from preexisting cells without involving cell proliferation as seen in hydra [5].

b) *Epimorphosis* - a phenomenon of regenerating or remodeling new tissue or organs, through the formation of blastema *e.g.* echinoderms [6] or non-blastemal routes *e.g.* liver and bone [7][8] in humans.

Cell proliferation, differentiation, dedifferentiation and transdifferentiation are involved during epimorphosis. These cellular activities involve synchronous molecular interactions between growth factors, chemokines or morphogens with the cell surface receptors and extracellular matrix (ECM) components (*e.g.* collagen, proteoglycans (PGs), fibronectin) that influence many biological processes beginning from embryonic development to homeostasis in adults. ECM and cell surface proteoglycans (PGs) with glycosaminoglycan [9] chains attached are important in regeneration or repair processes. They provide framework for cell activities like migration, maintenance at cell polarity for proper re-assembly of multilayer structures, and also binding and sequestering soluble proteins like growth factors and morphogens, thereby participating in various signaling pathways.

Adult limb regeneration has attracted considerable attention owing to the growing need in the reconstruction of structures such as cartilage, bone, nerves or blood vessels in patients suffering from degenerative or traumatic processes. Limbs are appendages that are involved in locomotion, feeding and communication in various organisms although they differ in morphological structures and shapes. A vertebrate limb is made of distinct cartilage and bone tissues. In invertebrates a limb is commonly termed as ‘appendage’, and does not have a strict organization of bone and cartilage. Nevertheless, vertebrate and invertebrate limbs share common functions like locomotion or feeding thus making them orthologous structures.
Brittlestar – An Invertebrate Model of Tissue Regeneration

The phylum Echinodermata (Greek: echinos – hedgehog, spine; derma – skin) contains small marine animals and is classified as shown in Figure 1.

![Taxonomic Classification of Phylum Echinodermata](image)

The most striking feature of echinoderms is their regeneration capacity that has attracted researchers to understand the different molecular mechanisms involved.

Brittlestars are characterized by a penta-radial symmetric disc and arms with spines and podia. Brittlestars are usually suspension or deposit feeders and arms are important appendages for their feeding behavior that are usually lost or autotomized (self-detachment) due to predation as a defensive function or hydrodynamic disturbance in their habitat [10]. Basic regeneration mechanism in brittlestar involves both morphallaxis and epimorphosis processes involving undifferentiated (coelomocytes) and differentiated (phagocytes and granule) cells [11]. The major advantage of using brittlestar as a model system is its simple anatomy, high regenerative capacity in a short time and their abundance in their natural habitat.

There is a growing amount of molecular information regarding the regeneration process of ophiruoids in particular brittlestar involving different growth factors and neuropeptides like Transforming growth factor β or bone morphogenetic protein (TGFβ/BMP), and SALMF amide [12-16] respectively. The information regarding the type of glycosaminoglycans (GAGs) or their interaction with any brittlestar relevant growth factors in relation to the re-
generation processes is still not known. Owing to the similarities in cellular and molecular mechanisms throughout the animal kingdom, brittlestar serves as a model to study the role of GAGs in a complex process of regeneration.
Proteoglycans and Glycosaminoglycans

PGs are proteins carrying polysaccharide chains known as GAGs that are covalently linked to their core. GAGs are linear polysaccharides consisting of repeated disaccharide units of a hexuronic acid and a hexosamine unit (Figure 2) that are attached to core proteins via O-linkage. The hexosamine unit is either N-acetylgalactosamine as in heparan sulfate (HS) / heparin (Hep) / hyaluronan (HA) / keratan sulfate (KS) or N-acetylglucosamine unit as in chondroitin sulfate (CS) and dermatan sulfate (DS). The hexuronic acid is D-glucuronate (GlcA) in all classes except KS, which contains galactose. Apart from HA, all GAG backbones are modified by epimerization of the GlcA to its C5 epimer L-idurionate (IdoA) (both in HS/Hep and CS/DS), N-sulfation (only in Hep and HS), O-sulfation, and occasionally other types of modifications such as e.g. fucosylation (CS/DS, KS) [17]. The modifications introduce an enormous structural heterogeneity to GAGs (detailed in section 'Chain modifications').

Figure 2. Disaccharide units of different GAGs. The hexosamine is galactosamine (dark grey) in CS/DS and glucosamine (light grey) in HS/Hep, HA and KS. Hexuronic acid is glucuronic acid (non shaded) in CS and HS, or its epimer iduronic acid (boxed) in DS and HS/Hep. KS contains a neutral galactose instead of the hexuronic acid unit. R₁ and R₂ indicate modifications of the C2 amine with either COCH₃ or SO₃⁻ and hydroxyls with SO₃⁻, respectively.
PGs exist throughout the animal kingdom from invertebrates to vertebrates. They can be classified either based on their core protein, the type of GAG chain attached to the protein (HSPG or CSPG) or their predominant site of expression. Based on their topological position in a cell or tissue, PGs can be classified as extracellular (e.g. Perlecan, Aggrecan, Versican, Decorin) membrane bound (e.g. Syndecan, Glypican) and intracellular (e.g. Serglycin) (Figure 3) [18, 19]. PGs can carry either one type of GAG chain or more than one GAG subtype. Agrin and decorin with HS and DS chains respectively are examples of the former type. Syndecan with HS and CS [20] and aggrecan with CS and KS [21] represent the latter type of PGs. Thus PGs introduce another level of structural heterogeneity and complexity.

Figure 3. Different topological position of PGs. HSPGs and CSPGs found intracellular e.g. serglycin, plasma membrane e.g. syndecan and in the extracellular matrix e.g. aggrecan.

PGs and GAG structures are expressed in a cell and tissue type specific manner [22], and are spatially and temporally regulated under different physiological and pathological conditions [23-25].

The biological importance of heterogeneous GAG structures and their PGs are demonstrated by a) gene targeting of GAG biosynthetic enzymes or PG core proteins in animal (e.g. mouse, Caenorhabditis elegans, Drosophila melanogaster) and in vitro cell models (e.g. Chinese hamster ovary (CHO) cells), and by b) reverse genetics (e.g. Danio rerio) [26, 27].
Biosynthesis of Glycosaminoglycans

The biosynthesis of GAG chains takes place in the Golgi compartments and involves a number of enzymes potentially organized in a multienzyme complex, called a GAGosome [28]. The complete biosynthesis can be described in three major steps, chain initiation, polymerization and modification. These steps happen more or less synchronously in the cell.

GAG biosynthesis is initiated on a target protein by xylosyltransferase (XylT), transferring a xylose from uridine -5’-diphosphate (UDP)-Xyl to a serine (Ser) residue within the protein [29]. Subsequent attachment of two galactose units (Gal) and a GlcA by galactosyltransferase I (GalT-I) and glucuronosyltransferase I (GlcAT-I), respectively, completes the linkage tetrasaccharide common to CS, DS, HS and Hep (Figure 4). Further modifications of this tetrasaccharide such as phosphorylation of Xyl or sulfation of Gal can regulate GAG biosynthesis as an early post-translational event [30] [31]. Mutational studies of these enzymes show the biological importance of the linkage region. Heterozygous mutations of enzymes such as XylT, GalT (I and II) and GlcAT in C.elegans produces severe defects in cytokinesis and vulval morphogeneis [32-35], while homozygous mutation in GalT I is embryonically lethal as in D. melanogaster [36, 37].

The HS and CS biosynthesis pathway diverge after this common tetrasaccharide linkage depending on the next sugar added. Addition of N-acetyl glucosamine (GlcNAc) by GlcNAc transferases (GlcNAcT) initiates HS/Hep chains, while transfer of N-acetyl galactosamine (GalNAc) by GalNAc transferases (GalNAcT) results in CS/DS chain biosynthesis [38]. The subsequent chain elongation and its modifications involve enzymes like polymerases, epimerases and sulfotransferases, UDP-sugars and the sulfate donor 3’-phosphoadenosine 5’-phosphosulfate (PAPS).

**PAPS Biosynthesis**

Sulfate groups are added to acceptor GAG chains from the universal sulfate donor PAPS. The sulfate donor is synthesized in the cytosol in a two-step coupled reaction by adenosine tri-phosphate-sulfurylase (ATP-sulfurylase) and adenosine 5’ phosphosulfate-kinase (APS-kinase) enzymes in the presence of magnesium (Mg^{2+}). In the first step, inorganic sulfate combines with ATP to form APS and pyrophosphate (PP_i) catalyzed by the ATP-sulfurylase enzyme. In the subsequent step ATP-kinase catalyzes the transfer of ATP to APS to form PAPS and adenosine di-phosphate (ADP) [39].
ATP-sulfurylase; Mg$^{2+}$ ATP + SO$_4^{2-}$ → APS + PP$_i$

APS-kinase; Mg$^{2+}$ APS + ATP → PAPS + ADP

In higher animals PAPS synthesis is catalyzed by PAPS-synthases (PAPSS), bifunctional proteins containing sulfurylase and kinase catalytic domains at the carboxy and amino terminal, respectively. After synthesis, PAPS is transported to Golgi compartments by PAPS-transporters where it serves as a sulfate donor for sulfotransferases. In Golgi, different chain specific sulfotransferases transfer sulfuryl group (SO$_3^-$) from the donor PAPS to an amino ($N$-sulfates, e.g. in HS) or a hydroxyl group ($O$-sulfates, e.g. HS and CS/DS) at specific position in a monosaccharide (see page 16 and 18).

Growing GAG chains (except hyaluronan) are the major acceptors of sulfates from PAPS, which is needed for their proper biological function. The importance of GAG sulfation in biological systems can be studied by inhibiting PAPS synthesis, and thereby sulfation, with sodium chlorate. Chlorate inhibits the formation of PAPS by competitively binding to the active site of PAPS-synthase as an analog [40, 41].

Heparan Sulfate/Heparin Biosynthesis

HS chain biosynthesis begins with alternating addition of UDP-sugars of GlcA and GlcNAc by one or more polymerases, the so called exostosins (Ext 1 and 2) in mammals. Chain modification starts with partial $N$-deacetylation and $N$-sulfation of GlcNAc residues by $N$-deacetylase / $N$-sulfotransferase (NDST) enzymes [43, 44]. Within the modified areas some of the GlcA residues are epimerized at C5 to form IdoA by uronyl C5-epimerase (Hsepi) and finally sulfates are attached in different positions by specific $O$-sulfotransferases (HSOSTs) on growing HS chains [45, 46]. To add to the complexity, endosulfatases (Sulfs) may remove 6-$O$-sulfate groups from the final product in the extracellular environment thus altering the chains and thereby affecting binding capacity for proteins [47]. Mice deficient with Ndst1, Hsepi, and HS2OST die shortly after birth with several developmental defects including renal agenesis in Hsepi$^{-/-}$ and HS2OST$^{-/-}$ [48-50].

Chondroitin Sulfate/Dermatan Sulfate Biosynthesis

The CS/DS chain synthesis involves polymerization and chain modifications (Figure 4) in the Golgi apparatus similar to HS biosynthesis.
Figure 4. Chondroitin sulfate/dermatan sulfate biosynthesis. The major steps of biosynthesis are polymerization and modifications (epimerization, O-sulfation). The disaccharide units to the left demonstrate typical examples of O-sulfation by the respective enzymes (modified structure highlighted).

Chain Polymerization

CS polymerization begins with synthesis of the tetrasaccharide linkage sequence followed by addition of alternating units of GlcA and GalNAc by glycosyl transferases from their respective UDP-sugar donors [38, 51]. The glycosyltransferases involved in chondroitin (Chn) polymerization are chondroitin synthases (CSS/ChSy) and chondroitin polymerizing factor (ChPF). These enzymes are ubiquitously expressed in all tissues and display sequence homology from invertebrates to vertebrates [52-56]. Mutation in C. elegans of either ChSy (sqv-5) or ChPF (pfc-1) results in defects in cytokinesis during early embryogenesis [34, 57]. So far six chondroitin glycosyltransferases have been identified: CSS1/ChSy-1, CSS2/ChPF, CSS3/ChSy-2, chondroitin sulfate glucuronyltransferase/ChSy-3, and chondroitin N-acetylgalactos-aminyltransferase-1 and -2 [52-54, 58, 59].

Chain Modifications

The following steps in CS/DS biosynthesis are modifications of the polysaccharide chains. These involve two major aspects: One is epimerization of the uronic acid unit (GlcA to IdoA) and the other is O-sulfation. Modifications
by specific enzymes are the key step in creating enormous structural diversity [43] in GAG chains in a cell and tissue specific manner [60].

Epimerization
While CS contains GlcA, epimerization by C5-epimerases Dsepi1 and Dsepi2 at position C5 converts CS to DS [61]. During DS biosynthesis epimerization of GlcA precedes O-sulfation [62]. IdoA provides structural flexibility to the DS chains imparting its functional role in protein binding [63] and the lack of such epimerized units as in DS-epi 1- mice, affects collagen fibril formation and thereby tensile strength of the skin [64].

O-Sulfation
This process involves the integrated action of three major players, a sulfotransferase, a sulfate donor (PAPS) and an acceptor molecule [9] in the Golgi compartment. O-Sulfates are attached to a number of potential carbon positions in GalNAc (C4, C6), GlcA and IdoA (C2, C3) through the action of specific O-sulfotransferases in the growing CS/DS chain (Figure 4) [43]. O-sulfation of CS/DS by different sulfotransferases are detailed below.

6-O-Sulfotransferase
Chondroitin 6-O-sulfotransferase (C6ST) modifies the hydroxyl in C6-position of GalNAc in CS chains (Figure 4) to produce GlcA-GalNAc(6S) (also called CSC unit). C6ST 1 and 2 are two isoforms that use CS as substrate and belong to the C6ST/KSGal6ST/GlcNAc6ST gene family [65-69]. A DS specific dermatan 6-O-sulfotransferase (D6ST) transfers a sulfate group to the C6-position of GalNAc residue flanked by IdoA units (IdoAα1-3GalNAcβ1-4IdoA) but not on CS chains or 4-O-sulfated DS units [70]. A non-mammalian DS unit (IdoAα1-3GalNAc(6S)) discovered in hagfish notochord and sea urchin indicates the presence of D6ST [71, 72] in such organisms. Mutation of the C6ST gene results in varied defects in different organisms, for example C6ST-1- mice show decreased naïve T lymphocytes in spleen [66]. In humans a missense mutation that changes a single amino acid (arginine to glutamine) within the PAPS binding site of the enzyme leads to spondyloepiphyseal dysplasia, characterized by severe skeletal malformations such as joint contractures, dystrophic dysplasia and spinal defects [73, 74].

4-O-Sulfotransferase
CS/DS disaccharides are frequently 4-O-sulfated through the action of 4-O-sulfotransferases which add sulfate groups to GalNAc residues adjacent to GlcA or IdoA (Figure 4). GalNAc(4S) prevents further epimerization of GlcA or IdoA. Three distinct chondroitin sulfotransferases (C4ST) -1, -2 and -3 encoded by CHST 11, -12, -13 genes, respectively, have been identified and cloned. All of these prefer GlcA rich chains generating GlcA-
GalNAc(4S) (CSA unit) [75-77]. Loss of function mutation of CHST 11 leads to improper growth factor signaling thereby resulting in chondroplasia. Intriguingly, elimination of C4ST not only decreases CSA (defined in Figure 5) content but also the amount of CS implying a feedback or regulatory role of C4ST in CS biosynthesis [78].

A DS specific dermatan 4-O-sulfotransferase encoded by gene CHST 14 catalyzes the transfer of sulfate at C4 of GalNAc residues adjacent to IdoA [79]. Adducted thumb-clubfoot syndrome is the first human congenital disorder with homozygous missense mutation of CHST 14 and is characterized by a connective tissue disorder with thumb and feet contractures. These sulfotransferases are grouped in the C4ST/D4ST gene family [80].

2-O-Sulfotransferase
Sulfates are added by the uronyl-2-O-sulfotransferase (CS/DS2ST) to GlcA or IdoA preceded by 6- or 4-O-sulfation of GalNAc in CS or DS, respectively (Figure 4). So far, only one CS/DS2ST belonging to the HS2ST/UA2OST gene family has been identified and cloned [81]. In DS, IdoA(2S) is observed frequently resulting in IdoA(2S)-GalNAc(4S) (CSiB or DS unit) rich structures important for the biological activity of these GAG chains.

Apart for the above stated modifications, CS/DS chains can be subject to further modifications e.g. fucosylation or glucose branching [17, 82] adding to the structural complexity. The nomenclature of CS/DS chains is based on the position of the sulfate groups. Non-mammalian sources are known to be rich sources for such odd CS/DS structures (see page 21).

The chain modification enzymes can either act alone resulting in monosulfated units e.g. CSA, or in combinations, leading to disulfated or trisulfated units e.g. DS or CST, thus producing a range of CS/DS units (Figure 5). The structural heterogeneity within the chain and also between different chains contribute to their structural complexity and thus form the basis for a wide range of biological functions [38]. Furthermore, sulfotransferases not only recognize the specific position in the target sugar unit but are also influenced by the structure of neighboring sugar units [83] thereby allowing fine regulation of modifications. As in all GAG biosynthesis, modifications are incomplete i.e. not all the substrate sites available are modified. In CS/DS chains domains of epimerized units (IdoA-GalNAc), non-epimerized units (GlcA-GalNAc), and mixed epimerized/non-epimerized structures are created [61]. This structural organization of chains in domains further provides a broader range of possible interactions for proteins (see page 23).
<table>
<thead>
<tr>
<th></th>
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<td><strong>Symbol</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
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<td>CSE</td>
<td>4S6S</td>
</tr>
<tr>
<td>2)</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>CSD</td>
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</tr>
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<td>CSB</td>
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</table>

*Modified from [28].

**Figure 5.** Different CS/DS structures found in the animal kingdom*. Symbol 1 indicates disaccharide abbreviation according to nomenclature [28] and 2 gives a shortened abbreviation used in this thesis. *Modified from [28].
Evolution of GAGs

GAGs are found in all multicellular organisms from invertebrates like nematodes (*C. elegans*) to higher vertebrates such as mammals [84]. Sulfated GAGs are absent in prokaryotes, but certain pathogenic bacteria (*e.g.* *Escherichia coli*, *Staphylococcus aureus*, *Neisseria meningitides*) contain linear polysaccharides of repeated units of N-acetylated amino sugars and uronic acid units similar to hyaluronan, non-sulfated heparin (heparosan) [85, 86] or non-sulfated chondroitin (chondrosan) [87]. Protista, plant and fungi lack GAG-like structures, while porifera (marine sponges belonging to basal metazoans) contain acidic glycans with uronic acid and hexosamine units, and other sulfated polysaccharides [88-90], but no classical GAGs.

Cássaro and Dietrich performed the first systematic analyses of GAG distribution in invertebrates [91]. These early approaches to systematically link GAGs to evolution were limited due to lack of analytical tools and sensitive methods for GAG analysis as well as species determination. The recent availability of recombinant, specific GAG glycosidases and improvement in sensitive analytical tools and methods for *e.g.* fluorescent tagging and high performance liquid chromatography (HPLC)[22, 92] or mass spectrometry (MS) techniques [93, 94] now allow us to characterize more heterogeneous GAG structures. The recent knowledge of distribution of GAGs in animal kingdom is shown in *Figure 6* [84].
Galactosaminoglycan Evolution

Galactosaminoglycans appeared in eumetazoa (organisms with true tissue structures) while DS emerged late in the GAG evolution in deuterostomes (Figure 6). CS and DS demonstrate vast structural heterogeneity across the phylogenetic tree [84, 95]. It can be reasoned that the need for CS chain epimerization to DS arose later due to some specific biological function, although GlcA to IdoA epimerization emerged with the occurrence of HS/Hep structures. Marine animals are known to be a rich source of GAG structures with unique modifications like sulfation at C3 of GlcA (GlcA3O) in king crab [96] or branching sugar units (e.g. fucose or glucose) in sea cucumber [97-100] when compared to higher vertebrates. Few other interesting CS/DS units discovered in marine species are CSU and CST in sea clam (*Scapharca inaequivalvis*) and CSH in hagfish (*Eptatretus burgeri*) [101, 102] (see Figure 5). Apart from such unique and diverse modifications found throughout the animal kingdom, CS/DS from mammalian sources e.g. humans are less sulfated [91] and CS from nematode e.g. *C. elegans* are non-sulfated (Chn)[103]. Thus, the abundance of GAG structures with enormous structural diversity suggests their significance for biological functions during the evolution of higher organisms.
GAG-Protein Interactions

As highlighted in the initial part of this thesis, GAGs are known to be involved in different physiological aspects during the life span of an organism. GAG/protein interactions are widely studied in the field of GAG biology in order to understand their structure-function relationship in different physiological aspects like ligand receptor binding, cell-cell cross talk, cellular architecture, or cytoskeletal interactions [23, 104, 105] (Figure 7).

![Diagram](image)

*Figure 7.* Different physiological roles of PGs and their GAG chains. They are engaged in ternary ligand-receptor complexes and may affect intracellular signaling (*i*). These molecules are in diverse manner involved in extracellular matrix architecture (*ii*), cell-cell cross talk (*iii*) and cytoskeletal interactions (*iv*).

GAGs interact with proteins mainly by ionic attraction between negatively charged sulfate groups and carboxy groups of GAG chains and basic amino acids of the protein, although some binding depends on non-ionic interaction.
The GAG domain (see page 17) structures allow for selective binding of protein ligands [61, 105, 107].

Of many GAG binding proteins, probably only a few require defined GAG sequences or sulfation patterns within the GAG chain, while the majority of them recognize less stringent sulfated domains. An example of sequence specificity is the interaction between the anticoagulant protease inhibitor antithrombin (ATIII) and heparin. ATIII recognizes and binds to the heparin sequence -GlcNAc6S-GlcA-GlcNS3S-IdoA2S-GlcNS- resulting in a conformational change of the protein. This in turn enhances binding of AT to thrombin, thereby affecting the coagulation cascade more efficiently [108]. Similar to ATIII, another anticoagulant enzyme, heparin cofactor II (HepC II), binds preferentially to a -IdoA(2S)-Hexosamine(4S) sequence in either DS or heparin thereby inhibiting coagulation [109] [110]. The structural flexibility of IdoA in GAG chains is considered to be one key property for their ability to interact with various protein ligands [111].

A well-studied example of a less stringent GAG/protein interactions is the binding of FGF to highly sulfated domains in HS or CS/DS and also to FGF receptors, thereby forming a stable and active ternary signaling complex [107, 112].

**Fibroblast Growth Factors, their Receptors and Intracellular Signaling**

FGFs, first identified in fibroblasts, are polypeptide mitogenic growth factors (17-34 KDa) found in all metazoan organisms *e.g.* invertebrates such as *C. elegans, D. melanogaster* [113, 114], echinodermata [115], and vertebrates like zebra fish and humans [116]. FGFs exert their biological effects by binding to high affinity transmembrane receptor tyrosine kinases (RTKs) known as FGF receptors (FGFR) and GAG chains. The GAG chains function as low affinity co-receptors that are required to induce intracellular signal cascades upon formation of a ternary complex [117]. FGFRs consist of three extracellular immunoglobulin (Ig) like domains (I-III), a transmembrane domain and two cytoplasmic tyrosine kinase domains (I-II) (*Figure 8A*). There are four different FGFRs (1-4) and FGFR1-3 all occur in splice variants (b and c) of domain III, thereby increasing the functional diversity of the FGF signaling system by affecting the ligand binding specificity [118].

Crystal structure studies have revealed two possible stoichiometric models of FGF-FGFR-HS ternary complexes, a symmetric model (2:2:2 of each component) with two HS chains engaged, and an asymmetric model (2:2:1) containing only one chain [119-122]. The length and overall sulfation of the HS
domain influence the binding and stability of the ternary complex and its downstream signaling [107, 123].

Figure 8. Schematic representation of a FGFR (A); FGF-FGFR-GAG ternary complex and intracellular signaling (exemplified by the MAPK-signaling pathway) (B).

**FGF Induced Signaling**

The FGF mediated signaling is regulated at the levels of growth factor expression, binding of FGF to its receptor, and FGF interaction with GAGs. Ternary complex formation at the cell surface leads to transphosphorylation of different tyrosine residues in the intracellular kinase domain creating a binding site for downstream signaling molecules. The best-studied FGF-induced signaling pathway, the Ras-Mitogen activated protein kinase (Ras-MAPK) pathway is outlined above (see Figure 8B).

Briefly, the cascade mainly involves protein interactions and activating or inhibiting phosphorylations. RTK phosphorylation at specific sites facilitates docking of the FR substrate 2 (FRS2) to the activated receptor. That in turn results in phosphorylation of FRS2, and subsequent recruitment of the Grb2/SOS (Son of Seven less) complex and phosphorylation of the downstream effector Raf. Activated Raf then phosphorylates MAPK/extracellular signal-regulated kinases 1/2 (MEK1/2), which subsequently phosphorylate extracellular signal regulated kinases 1 and 2 (ERK1/2). Activated ERK1/2 phosphorylate a variety of cytoplasmic substrates and also translocate to the nucleus where they activate transcription factors resulting in mitogenesis [124].
Bone Morphogenetic Proteins

Another well-studied family of GAG interacting proteins are the Bone morphogenetic proteins (BMPs). BMPs belong to the transforming growth factor β (TGFβ) superfamily. These growth factors are widely represented throughout the animal kingdom from invertebrates [15, 125] to vertebrates and they have been implicated in a variety of developmental processes. BMPs are dimeric molecules composed of two 12-15 KDa polypeptide chains linked by a single interchain disulfide bond. There are >30 known members of this family that is subdivided into several groups based on their structure and function [126].

As implied by their name, BMPs promote and regulate development, growth, morphogenesis, remodeling and repair of bone and cartilage. Besides this, BMPs are expressed spatiotemporally with a wide range of effects beginning from embryonic developmental processes including cell fate determination, chemotaxis, mitosis, differentiation, to postnatal growth of tissue structures such as eye, heart, kidney, or skin. Intriguingly, the biological activities of BMPs are not identical among their members as they bind to their receptors with different affinities [126].

The TGFβ superfamily of proteins is known to interact with HS/Hep and CS/DS and modulate different cellular responses [127-130]. Although attempts have been made to study the structural characteristics of GAGs that are responsible for binding to BMPs [131], much remains to be done in this area.
Present investigations

Regenerative medicine is an important field due to the growing need for tissue regeneration or reconstruction of cartilage, bones or nerves in patients with traumatic injury or disorder. The cellular and molecular mechanisms involved in regeneration have not been well elucidated. This is mainly due to the complexities in cell/tissue types and also to cell feedback mechanisms such as checkpoints. Hence regenerative capacity varies throughout the animal kingdom and has been explored only in a few model organisms.

Although GAGs have been studied in various physiological and pathological processes, not much is known about the influence of the heterogeneous GAGs in the complex process of regeneration. Structural analysis of the GAGs is the key to understand their biological functions.

Work in this thesis is aimed to understand the structure-function relationship of GAGs during regeneration in an invertebrate brittlestar model (Ophiuroidea). In order to achieve this goal, three different objectives were addressed:

- To characterize GAG structures in brittlestar species and determine their functional role in protein binding and cell signaling.
- To study the structure-function relationship of unique GAG structures in brittlestar *Amphiura filiformis* arm regeneration.
- To gain insight into gene expression of CS/DS biosynthetic enzymes during regeneration.
Results and Discussion

GAGs are biomolecules synthesized in a non-template driven manner. Enormous structural heterogeneity of GAGs imparted by several modifications on a simple alternating backbone of hexosamine and hexuronic acid, is the basis for their biological role throughout the animal kingdom. In an attempt to understand the structural heterogeneity and functional role of GAGs during evolution we used brittlestars as a model organism of high regenerative capacity.

Brittlestars Contain Highly Sulfated CS/DS that Promote FGF2 Induced Cell Signaling (Manuscript I)

An evolutionary view of GAG structural complexity reveals that lower animals, in particular invertebrates, have exceedingly oversulfated GAGs, along with other modifications adding to structural complexity. Our structural characterization of GAGs in brittlestars, showed that the polyanionic macromolecules were sensitive to enzymatic digestion with chondroitinase ABC (CS:ase ABC) while insensitive to selective chemical (HNO₂, pH 1.5) cleavage indicating the lack of N-sulfated glucosamine structures (HS/Hep) [63]. Chondroitinases (polysaccharide lyases) selectively cleave most of the CS/DS and HA by a β-elimination mechanism yielding mainly oligosaccharides (2-mer, 4-6-mers) with Δ4,5-unsaturated uronic acid (see Figure 9) [132]. HS/Hep and galactosaminoglycans with other branching modifications e.g. fucose are not cleaved by these enzymes [133]. Earlier studies on GAG structures from echinoderms like sea urchin, sea cucumber or feather star had identified HS [134] or CS/DS with fucose modification (Paracentrotus lividus), [98, 135, 136]. Here, we could isolate CS/DS with ~1.7 sulfate groups/disaccharide as the major GAG and no detectable amounts of HS in brittlestars. This is a remarkable finding since HS and heparin are known to appear at the beginning of the eumetazoan line and are conserved throughout evolution of multicellular organisms [84, 137]. Brittlestars in general and A. filiformis in particular possess higher amounts of CS/DS trisulfated disaccharides thus contributing to a remarkably high sulfation content (~ 2.3 sulfates/disaccharides), when compared not only to mammals.
[111], but also to other marine species e.g. ascidians [138] or hagfish notochord [139]. The high degree of sulfation in marine animals has been tentatively explained in terms of adaptation to their habitat i.e. a surrounding of high salinity [140].

Disaccharide analysis prompted us to further characterize the GAG chains of brittlestars (mixed species and A.filiormis) to look for the presence of organized domains of CS/DS units. Enzymatic cleavage using CS:ase ABC results in loss of information of IdoA [141] as this enzyme treatment produces disaccharides with unsaturated hexuronic acid units (Δ4,5HexA). To overcome this problem, we subjected brittlestar GAGs to restrictive cleavage using various chondroitin lyases. CS:ase ACII is an exolyase that cleaves non-epimerized, monosulfated disaccharides from the non-reducing end (Figure 9) [142-144]. Digestion of brittlestar CS/DS resulted in the release of CSA disaccharides, suggesting that there is a contiguous stretch of GlcA-GalNAc4S units at the non-reducing end of CS/DS polysaccharide. Cleavage with CS:ase B, which is an endolyase that cleaves 2-O-sulfated epimerized DS disaccharides, which are 4-O-sulfated and may be 6-O-sulfated, resulted in the release of a large portion of DS disaccharides. Combining the two enzymes resulted in the same amount of generated disaccharides as the sum of the individual treatments, but none of the residual saccharides obtained by CS:ase ABC treatment (Figure 9). This suggests the residual units are arranged in an alternating manner of epimerized and non-epimerized units and thus resistant to ACII and B enzymes. Taken together, these results demonstrate that brittlestar CS/DS contains domains of CS:ase B sensitive structures, CS:ase ACII sensitive and CS:ase ACII+B resistant structures. Thus our findings show that highly sulfated brittlestar CS/DS are organized in domains akin to those in HS [23].
GAGs are known to bind and sequester various basic proteins e.g. growth factors, chemokines, morphogens, and can therefore influence various signaling pathways [23, 105, 107]. To further elucidate the functional role of brittlestar CS/DS, we studied their ability to bind various proteins like FGFs (1 and 2), VEGF-A\textsubscript{165} and VEGF-A\textsubscript{165b} (lacks HS binding domain) and ATIII. Binding of FGFs and VEGF-A\textsubscript{165} to brittlestar CS/DS was similar to that of other CS/DS hybrid structures from invertebrates [105]. However, poor or no binding of ATIII and VEGF-A\textsubscript{165b} was observed, contradictory to earlier reports on invertebrate structures that facilitate ATIII binding [98]. This is probably due to the absence of fucose branching in brittlestar GAGs, previously shown to be important for ATIII binding [147]. CS/DS from marine species are known to promote neurite outgrowth [139] and angiogenesis [135] by binding to FGF2 and its receptor. In accordance to earlier studies, our findings also demonstrate that sulfated domains of brittlestar CS/DS promote FGF2 binding and thereby mediate effective signal transduction. This may indicate a possible role of sulfated domains for protein binding and their engagement in cell-regulatory mechanisms that can be extrapolated to regeneration in general.
A Potential Role for CS/DS in Arm Regeneration in *Amphiura filiformis* (Manuscript II)

Histochemical staining using alcian blue (a cationic dye) and phage display antibody (LKN1) specific for DS epitopes were performed to localize CS/DS in *A. filiformis* arms. Sulfated polysaccharides, as detected by alcian blue, were localized close to the arm wall, lining of podia, muscle and coelomic cavity. LKN1 staining showed a similar localization as alcian blue staining and could be abrogated by CS:ase ABC treatment. These results suggest that a majority of CS/DS is localized in the ECM close to different arm tissues. GAGs are known to be present on the cell surface and in the ECM. Previous anatomical and biochemical studies of intersegmental ECM in echinoderms have shown CS/DSPGs associated with collagenous tissue, suggested to be important during arm autotomy (self detachment) [10, 148, 149]. In vertebrates, DSPGs are shown to involve in wound repair by binding to growth factor such as FGF2 [150, 151]. The role of CS/DS in wound healing or repair in echinoderms is however not yet known. The CS/DS localization in *A. filiformis* arm and previous studies on CS/DSPGs in wound repair may suggest their participation in regeneration.

Further studies were performed to investigate whether CS/DS structures affect arm regeneration. For this purpose, experimental autotomy was induced in arms and the regenerates were collected at the blastema stage (1 week post amputation (p.a.)), at an ~50 % differentiation index (DI) stage (D3 weeks p.a.) and at ~95 % DI stage (5 weeks p.a.) for GAG isolation followed by disaccharide analysis. DI (as percentage) is calculated as the proportion in length of regenerate that is completely differentiated (as indicated by fully developed spines and podia). The arm regenerates were collected to characterize their GAGs. Structural characterization at different stages of regeneration showed an increase in trisulfated disaccharides with progression of regeneration when compared to the non-regenerated arm. As regeneration in *A. filiformis* is nerve dependent [11, 12] and oversulfated CS/DS from marine sources are known to promote neuro-regulatory activities and neuritogenesis [101, 152], these highly sulfated CS/DS may play a role in *A. filiformis* regeneration mechanisms.

In order to study the role of CS/DS in arm regeneration we employed the sulfate inhibitor sodium chlorate. Sodium chlorate competitively inhibits ATP-sulfurylase, an enzyme involved in PAPS (sulfate donor) synthesis [153]. Reduced PAPS levels lead to under-sulfation of GAGs. Our studies showed that the sodium chlorate concentration is inversely related to the regeneration capacity when compared to normal regeneration under chlorate free conditions (*Figure 10*). Proliferation and differentiation occur simulta-
neously during arm regeneration in *A. filiformis* [154]. Chlorate treatment affected the growth rate (length of the regenerates) more severely than differentiation (arms with well developed podia and spines) indicating an impact of chlorate on proliferation rather than on differentiation. This is in agreement with similar studies on cultured mammalian cells, which have shown that dampening of sulfation by chlorate treatment affects proliferation rates [155, 156]. Similar chlorate treatment studies in sea urchin (*Strongylocentrotus purpuratus*) embryo also showed severe developmental defects during their oral-aboral axial patterning and gastrulation due to lack of modified GAG structures [157]. Our results are in accordance with the above findings and highlight the importance of GAG sulfation in proliferation.

![Figure 10. Schematic presentation of *A. filiformis* arm regeneration at different chlorate concentrations. After induced arm autotomy the animals were kept at different sodium chlorate concentrations (sulfate inhibitor). Regeneration was followed by measuring growth rates and determining the differentiation index. The sagittal view of the arm showing coelomic cavity (c), muscle (m), radial water canal (rwc), nerve (n), podia (p), and spine (s) adapted from [11].](image)

**Gene Expression of CS/DS Biosynthetic Enzymes during Regeneration in *Amphiura filiformis* (Manuscript III)**

We have identified highly sulfated CS/DS type of GAGs in brittlestars, in particular in *A. filiformis* (Manuscript I) with structural changes apparent during the regeneration stages, which may participate in arm regeneration.
Further we addressed, whether CS/DS structure correlate with gene expression of their biosynthetic enzymes? For this purpose we selected three candidate CS/DS genes and named them *A. filiformis* chondroitin sulfate synthase-1 (*AfChSy1*), chondroitin 4O-sulfotransferase-2 (*AfC4ST-2*) and dermatan 4O-sulfotransferase (*AfD4ST*). A homology search of the *A. filiformis* transcriptome against CS/DS genes from different species was performed and primers were designed to the conserved region. In parallel we also analyzed BMP2/4 (*Afbmp2/4*), a known growth factor in *A. filiformis* arm regeneration [16].

The expression profile of the CS polymerase (*AfChsy1*) sulfotransferases (*AfC4ST-2 and AfD4ST*) was compared with structural changes observed at the product level. An increased gene expression of *AfChsy1* and *AfC4ST-2* at the 50% DI stage correlated with increased sulfation of the product. *AfD4ST* gene expression was downregulated even though 4-O-sulfation increased during regeneration. However, C4ST-2 and D4ST-1 are related enzymes that share sequence homology (22.8%) and have similar substrate preferences; As demonstrated for human enzymes, C4ST-2 transfers a sulfate to the 4-O-position of GalNAc in –GlcA-GalNAc-GlcA- or –IdoA-GalNAc-IdoA stretches, while D4ST-1 only recognizes the latter unit [79] [159]. Thus, it is possible that C4ST-2 alone is responsible for the increase in 4-O-sulfation, whether it occurs on GlcA or IdoA containing disaccharides. At 95% DI the arms have well developed nervous system as well as externally differentiated structures such as spines and podia representing a functional arm [154]. Increase in tri-O-sulfated disaccharide unit at 95% DI did not correspond to the sulfotransferase gene expression at that stage. This may be due to well-developed tissue structures with proper ECM architecture at later stage of regeneration and further synthesis of CS/DS is restricted in order to maintain the existing structures either by turnover or product inhibition as a feedback mechanism. The *Afbmp2/4* gene expression profile obtained is found in earlier studies in *A. filiformis* arms at different regeneration stages [15, 16, 125].

Highly sulfated CS/DS is known to bind many proteins such as FGF2, pleiotrophin, midkine [105], BMP2 or 4 [160, 161] and exert various cellular activities[152]. An *in vitro* study in MC3T3-E1 osteoblastic cells using over-sulfated CSE isolated from squid cartilage and BMP4 show enhanced cell growth, collagen deposition, alkaline phosphatase activity and mineralization during osteoblast differentiation [161]. Another study using gene trap approach has shown TGFβ/BMP to have a regulatory role on gene expression of *C4ST* and influence its spatio-temporal expression during differentiation in mouse embryogenesis [162-164]. From our results so far, *Afbmp2/4* expression shows a similar trend as the expression of *AfChsy1* and *AfC4ST-2*.
Thus our findings may suggest a functional correlation of ligand levels (BMP2/4) and its potential interaction partners (CS/DS) during regeneration.
A considerable fraction of ECM and cell membrane glycoconjugates are of GAG type, found throughout vertebrates and invertebrates. HS/Hep has dominated in GAG research owing to their potential biological function. In recent years CS/DS is gaining importance with advances in structural characterization and demonstration of its functional importance in processes such as wound repair and tissue engineering. CS/DS from invertebrates, provide a wide structural choice of products due to their heterogeneous nature. Further, such structures have potential therapeutic applications as alternatives to HS and heparin.

In this study, we have identified unique CS/DS structures from brittlestars with a high sulfation degree, protein binding and receptor stimulating capacity. We have also identified changes in CS/DS structures during regeneration and observed hampered regeneration during chlorate-induced impairment of sulfation. Furthermore, the gene expression study provides a starting point to understand the potential regulatory role of the CS/DS biosynthetic machinery that contributes to structural changes during the regeneration process. Thus these findings suggest a possible role of CS/DS in *A. filiformis* limb regeneration.

Regenerative medicine is a promising field and refers to a group of biomedical approaches like cell therapies using stem cells and tissue engineering. CS/DS is known to be an important GAG in limb development and repair. Recent research in limb tissue engineering is focused on building new scaffolds or delivery of nanoparticles consisting of CS/DS and other components, not only as supporting frame for cells but also as biological cues for promoting regeneration process by interacting with proteins. This is the first study to correlate a possible involvement of CS/DS in echinodermal limb regeneration. Further studies are required to gain more insight into the biological role of *A. filiformis* CS/DS by using different models like cell (*in vitro*) and mammalian (mouse) systems to answer the following questions. How does highly sulfated *A. filiformis* CS/DS regulate cell proliferation and thereby the regeneration process? Does CS/DS biosynthetic enzyme expression correlate to different structures produced during regeneration? Hence, this study is just the tip of the iceberg, opening a new path towards finding potential molecules from invertebrates having therapeutic relevance in regenerative medicine.
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