1 Aggrecan and Hyaluronan: The Infamous Cartilage Polyelectrolytes – Then and Now

Anna H. K. Plaas, Meghan M. Moran, John D. Sandy, and Vincent C. Hascall

Abstract

Cartilages are unique in the family of connective tissues in that they contain a high concentration of the glycosaminoglycans, chondroitin sulfate and keratan sulfate attached to the core protein of the proteoglycan, aggrecan. Multiple aggrecan molecules are organized in the extracellular matrix via a domain-specific molecular interaction with hyaluronan and a link protein, and these high molecular weight aggregates are immobilized within the collagen and glycoprotein network. The high negative charge density of glycosaminoglycans provides hydrophilicity, high osmotic swelling pressure and conformational flexibility, which together function to absorb fluctuations in biomechanical stresses on cartilage during movement of an articular joint. We have summarized information on the history and current knowledge obtained by biochemical and genetic approaches, on cell-mediated regulation of aggrecan metabolism and its role in skeletal development, growth as well as during the development of joint disease. In addition, we describe the pathways for hyaluronan metabolism, with particular focus on the role as a "metabolic rheostat" during chondrocyte responses in cartilage remodeling in growth and disease.

Future advances in effective therapeutic targeting of cartilage loss during osteoarthritic diseases of the joint as an organ as well as in cartilage tissue engineering would benefit from 'big data' approaches and bioinformatics, to uncover novel feed-forward and feed-back mechanisms for regulating transcription and translation of genes and their integration into cell-specific pathways.

Keywords

Cartilage · Aggrecan · Hyaluronan · Extracellular matrix

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1.1 Introduction

Cartilages are unique in the family of connective tissues in that they contain a high concentration of the glycosaminoglycans (GAG),

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chondroitin sulfate (CS) and keratan sulfate (KS) that are attached to the core protein of the proteoglycan, aggrecan. Aggrecan is organized in the extracellular matrix via a domain-specific molecular interaction with hyaluronan (HA) and with a link protein, and it is present throughout the collagen and glycoprotein network.

The high concentration of these organized GAGs have a well-documented essential role for articular cartilages to absorb alterations in biomechanical stresses during movement of an articular joint. At the structural level this is due to their biophysical characteristics at physiological pH, which include hydrophilicity and high osmotic swelling pressure due to the negative charges on their carbohydrate subunits (carboxyl and sulfate groups) and on their conformational flexibility and efficiency at filling space due to their sizes.

In this chapter we review the history and current knowledge of the cell-mediated regulation of aggrecan metabolism (Fig. 1.1) including: (a) the posttranslational modification of the core protein with CS and KS and its extracellular organization into 'aggregates' with HA and link proteins; (b) the proteolytic processing of the core protein by a specific set of extracellular proteases (ADAMTSs and MMPs); and (c) the function of hyaluronan (HA) metabolism in the context of serving as a "metabolic rheostat" during chondrocyte responses in cartilage remodeling during growth and disease.

Throughout the Chapter, components of the metabolic pathways that have been shown to be affected by biomechanical perturbation of tissues will be highlighted. In this research area, the Grodzinsky lab, together with an extensive network of collaborators, spearheaded in vitro bioreactor experiments using cartilage explants or chondrocytic cell constructs, to delineate the effects of static and dynamic compression, and of sheer stress, on the illustrated pathways in aggrecan post-translational processing. This set in motion a research approach used by multiple laboratories to extend our understanding of mechanotransduction pathways in chondrocytes and progenitor cells for cartilage engineering purposes ([77, 106, 144, 228, 239, 279] and references therein). In addition, throughout the comprehensive list of key references in the covered research areas the publications from the Grodzinsky lab and its past members are annotated in the Bibliography.

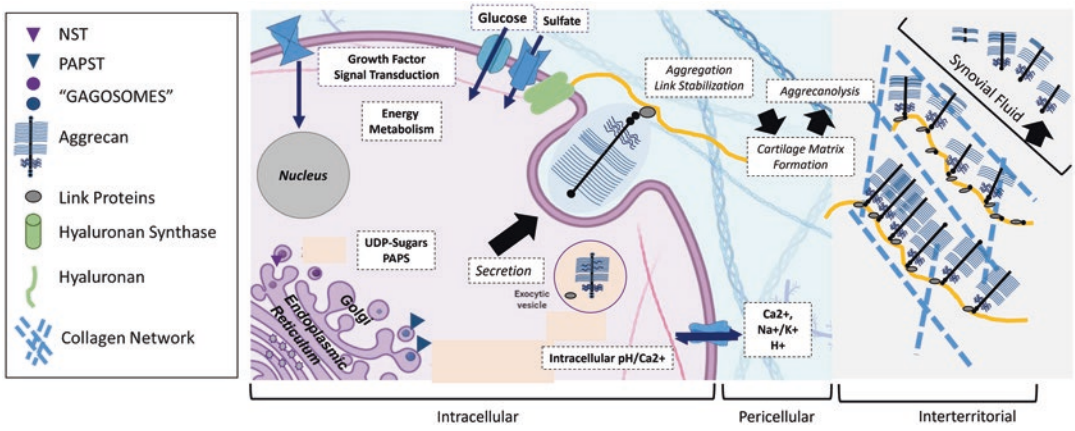

Fig. 1.1 Schematic of topographical organization of components involved in intracellular aggrecan synthesis and extracellular matrix organization

1.2 Chondroitin Sulfate and Keratan Sulfate Fine Structure on Aggrecan

Core protein linkage regions for synthesis and polymerization of CS and KS on the aggrecan core protein domains are illustrated in Fig. 1.2. CS is O-glycosidically linked to the serine residues along the CS rich regions 1 and 2 of the core protein via a linkage region oligosaccharide (-Xyl-Gal-Gal-GlcA) followed by unbranched chains consisting of disaccharides, (→4)β-GlcA (1→3)βGalNAc(1→), in which the amino sugar can be substituted on the C4 and/or C6 by a sulfate ester.

KS on aggrecan, also known as 'skeletal' KS, [180, 214] is O-linked to a serine or threonine in the KS domain, via a mucin core-2 linkage structure, (-GalNAc β(1–6)GlcNAc(1→). The GAG polymer is based on a polylactosamine backbone, with repeated disaccharides of (→4) βGal β (1–3) GlcNAc (1→). Both sugars in the disaccharide repeat can be sulfated on their C6 carbon, and an additional fucose can be substituted on the GlcNAc-6S. Many of these chains also capped with a sialic acid at the non-reducing terminal.

1.2.1 Aggrecan CS Chain Length and Sulfation Are Different in Skeletal Growth and Mature Cartilages

It is well established that chain length of CS and the type of sulfation on the C-4 or C-6 position of GalNAc residues in CS can vary with cartilage source depending on species and anatomical location. Detailed analyses of aggrecan CS fine structures in cartilage growth and maturation have provided more insights into conserved adaptations of CS biosynthesis to altered biophysical and biomechanical demands of a particular cartilage type.

Thus, examination of the GAG fine structure on growth and mature cartilage aggrecan core protein GAG domains using HPLC [163, 199]

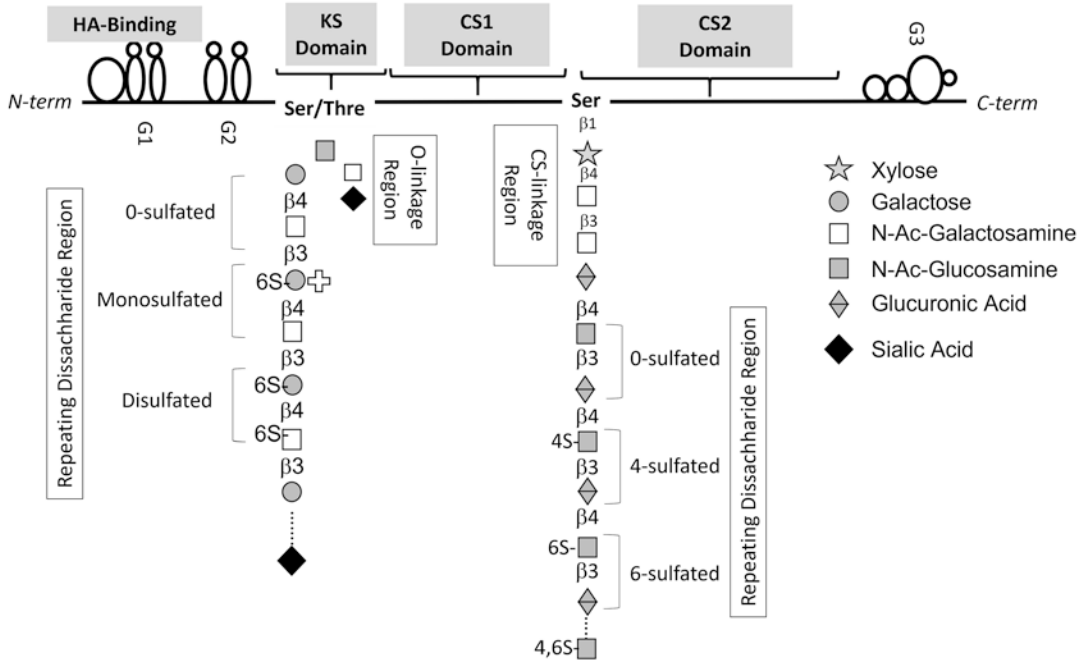

Fig. 1.2 Schematic of Aggrecan Core Protein Domains: G1, HA binding; KS, KS or O-linked oligosaccharide substituted domain, CS1/CS2, CS attachment domains

and FACE analyses [33, 201] established both location and age-related changes. For example, CS fine structure analyses of fetal growth plate cartilage aggrecan revealed a gradient in CS composition from the reserve zone to the hypertrophic zone, characterized by a marked increase in chain length accompanied by increased 6-sulfation and a concomitant decrease in 4-sulfation [55]. Furthermore, major changes in both CS chain length and sulfation pattern during postnatal maturation of human knee cartilage from the epiphyseal growth to a mature articular phenotype [200, 214, 285] were also detected. Upon skeletal maturation, chain length decreased by as much as 50%, and transitioned from an equal abundance of 4- and 6-sulfated GalNAc residues in growth cartilage to a predominance of 6-sulfated GalNAc residues. In addition CS chains in the CS2 region were shorter than those in the CS1 domain and carried a non-reducing terminal 4, 6-disulfated GalNAc residue instead of a 4S-GalNAc residue. A similar pattern in decreased chain length and increased 6-sulfation of both internal and terminal GalNAc residues was also observed by analyses of equine carpal articular cartilage CS [27].

1.2.2 GAG Biosynthesis Is a Multienzyme Process That Takes Place During Core Protein Trafficking Through the ER and Golgi

Studies to-date have shown that the conserved heterogeneity in GAG fine structures, unlike protein synthesis, do not follow a template, and it is regulated by individual cell phenotypes as well as by the structure of the proteoglycan core proteins that provide the acceptors. It is now recognized that conserved GAG structures are generated by transcriptional [124, 164, 288] and topographical [127, 238, 248, 249] control of the numerous enzymes responsible for linkage region synthesis and by GAG polymerization and sulfation (Table 1.1).

1.2.3 Skeletal Disorders Caused by Defective Genes Encoding Biosynthetic Enzymes for Sulfated Glycosaminoglycans

The generation of knock out mouse strains deficient in these enzymes revealed that many had an embryonic lethal phenotype due to defective cell proliferation and organ development, or altered neuronal function. However, they did not reveal a specific function for their role in cartilage growth and maturation (Table 1.2). On the other hand, human genetic studies revealed that defects in GAG-biosynthetic glycosyltransferases, epimerases or sulfotransferases cause distinct phenotypes of congenital disorders in cartilage growth, such as skeletal dysplasia, chondrodysplasia, multiple exostoses, and Ehlers-Danlos syndrome. This has furthered our understanding of the functional importance in the CS substitution on the aggrecan core protein (Table 1.3). In addition to the studies listed, individuals with either Kashin–Beck disease (KBD) [84], who show a dysfunction of CS sulfation enzymes, or a rare polymorphism in the aggrecan core protein [61, 122] are pre-disposed to the development of multi-joint or hand osteoarthritis, respectively.

1.2.4 Intracellular Localization and Topographical Organization of Enzymes for Aggrecan GAG Synthesis

The initiation of the linkage region by xylosyltransferases (I or II) [80, 203, 247] using UDP-xylose for addition of xylose to CS-region serine residues on aggrecan has been shown to occur in a pre-Golgi compartment, either at endoplasmic reticulum (ER) exit sites or in the ER-Golgi intermediate compartment [115, 174, 267]. However, the locations of these enzymes are also proteoglycan core protein and/or cell type specific since xylosyltransferases (I and II) were identified in the cis-Golgi region in rat liver cells and

Table 1.1 Chondroitin sulfate synthetic enzymes

Enzyme	Human gene name https://www.ncbi.nlm.nih.gov/gene	Human mRNA accession #	Gene records for other species
Linkage region			
Xylosyltransferase 1 (XylT-1)	*XYLT1*	NM_022167	Mouse, Rat, Dog, Pig
Xylosyltransferase 2 (XylT-2)	*XYLT2*	NM_007255	Mouse, Rat, Dog, Bovine
Beta-1,4-Galactosyltransferase 1 (GalT-I)	*B4GALT7*	NM_080605	Mouse, Rat
Beta-1,4-Galactosyltransferase 2 (GalT-II)	*B3GALT6*	NM_012200	Mouse, Rat, Pig
Beta-1,3-Glucuronyltransferase 1 (GlcAT-I)	*B3GAT3*	NM_014864	Mouse, Rat, Pig
Repeating disaccharide region			
Beta-1,4-Glucuronyltransferase 1 (GlcAT-II) Beta-1,3 NAcetyl Galactosaminyl transferase II (GalNAcTII)	*CHSY1* *CHSY2 (CSS3)* *CHSY3 (CHPF2)*	NM_014918 NM_175856 NM_019015	Mouse, Rat, Bovine — Mouse, Rat, Bovine
Chondroitin Polymerizing Factor (GalNAcT-II, CS-GlcAT-II)	*CHPF (CSS2)*	NM_024536	Mouse, Rat
Chondroitin N-GalNAc transferase (GalNAcT-I; GalNAcT-II)	*CSGALNACT1* *CSGALNACT2*	NM_018371 NM_018590	Mouse, Rat, Bovine, Pig, Horse Mouse, Rat, Bovine, Pig, Horse
Chondroitin 4-O-Sulfotransferase	*CHST11 (C4ST-1)* *CHST12 (C4ST-2)* *CHST13 (C4ST-3)*	NM_018413 NM_018641 NM_152889	Mouse, Rat Mouse, Rat, Bovine, Pig, Horse Mouse, Rat, Bovine, Pig
Chondroitin 6-O-Sulfotransferase	*CHST3 (C6ST-1)*	NM_004273	Mouse, Rat, Bovine, Pig, Horse
N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase	*CHST15*	NM_015892	Mouse, Rat, Bovine, Pig

chondrosarcoma cells [149, 181]. Glycosyl- and sulfotransferases for extension and sulfation of the CS chains in the C4 or C6 positon of the GalNAc residues takes place in the Golgi stacks and extends into the trans-Golgi network (TGN) [249, 264].

Much less is known about the topographical location of the O-linked KS synthesis enzymes, largely impeded by the fact that their activity rapidly declines when tissues or cells are maintained ex vivo [75, 179]. For example, it has not been determined whether CS and KS synthesis occur simultaneously or whether GAG-specific enzymes are segregated in Golgi subcompartments, or whether there is a regulated temporal recruitment as the core protein is trafficked through the secretory pathway enzymes in the same compartment.

Table 1.2 Genetic deletion of CS-synthesis enzymes in mice and associated phenotypes

Enzyme	Knock-out Mouse strains	Major phenotype
Linkage region		
Xylosyltransferase 2	Ferencz et al. [69]	Increased weight differences of lung, heart, and spleen.
Beta-1,4-Galactosyltransferase 1	Kido et al. [117] and Nakamura et al. [178]	Altered brain development
Beta-1,4-Galactosyltransferase 2	Asano et al. [7]	Defective proliferation and differentiation of epithelial cells; growth retardation Embryonic lethality, growth retardation
Beta-1,3-Glucuronyltransferase 1	Izumikawa et al. [108], Yada et al. [290], and Gotoh et al. [79]	Embryonic lethality due to failed cytokinesis
Repeating disaccharide region		
Chondroitin N-GalNAc transferase	Inada et al. [104], Watanabe et al. [278], Sato et al. [226], Shimbo et al. [234], and Adhikara et al. [1]	Defective neuronal plasticity and axon regeneration Defective cartilage growth and collagen organization; defective enchondral ossification; chondrodysplasia; impaired macrophage action
Chondroitin 6-O-Sulfotransferase	Ito et al. [107]	Enhanced motor function recovery after spinal cord injury
Chondroitin 4-O-Sulfotransferase	**Not available**	Abnormal CS elongation shown in sog9 murine L cell mutant
GalNAc4-Sulfate 6-O-Sulfotransferase	Habuchi et al. [83], Kitazawa et al. [126] and Ohtake-Niimi et al. [186]	Enhanced liver fibrosis; abnormal perineuronal net; altered bone marrow derived mast cells; altered dermal repair

Table 1.3 Human skeletal disorders caused by genetic abnormalities in CS-synthesis

Gene/protein	MIM No	Clinical features of resulting skeletal defects
XYLT1 (Xylosyl transferase 1) Desbuquios dysplasia type II Baratola Scott syndrome	**61577** **608124** **300681**	Short stature, joint laxity, hand abnormalities Short stature, patellar dislocation, facial abnormalities
SLC26A2 (Sulfate Transporter) Achondrogenesis type IB Diastrophic Dyplasia Multiple Epiphyseal Dysplasia	**600972** **222600** **226900**	Pre- or early post-natal lethal chondrodysplasia with underdeveloped skeleton Epiphyseal Dysplasia, early onset of Osteoarthritis
PAPSS2 (PAPS Synthase-2) Spondyloepimetaphyseal dysplasia	**612847**	Short bowed lower limbs, enlarged knee joints, short trunk, scoliosis
SLC35D1 (UDP-GlcA/UDP-GalNAc transporter) Schneckbecken dysplasia	**269250**	Neonatal lethal chondrodysplasia short long bones, deformed vertebral bodies
B4GALT7 (GalT-I) EDS, progeroid form	**130070**	Short stature, cranial dysmorphism, osteopenia, aged appearance
B3GALT6 (GalT-II) Ehlers Danlos Syndrome Spondylodysplatic type 2	**615349** **615291**	Short stature, joint laxity and dislocation, spondylodysplasia
B3GAT3 (GlcAT-I) Larsen-like Syndrome	**245600**	Joint dislocations mainly at elbow, scoliosis
CHSY1 (Chondroitin Synthase 1) Temtamy preaxial brachydactyl syndrome	**605282** **608183**	Short stature, limb malformations, growth retardation
CSGALNACT1 (GalNAcT-II, Mild Skeletal Dysplasia	**616615**	Brachydoctyly, joint lacity, mild facial deformations
CHST3 (CS6 sulfotransferase) Spondyloepiphyseal dysplasia	**143095** **603799**	Short stature, dislocation of large joints, kyphoscoliosis, osteoarthritis of elbow, wrist and knee
CHT11 (CS4 sulfotransferase) Osteochondrodysplasia brachydactyly	**610128** **618167**	Brachydactyly, clinosymphalangism in hands and feet, syndactyly and hexadactyly in feet, scoliosis, dislocated patellae, and fibulae and pectus

1.2.5 ER/Golgi Topography and Organelle Microenvironment of GAG Synthesizing Enzymes

The ER/Golgi membrane localization of the GAG synthesis enzymes has been confirmed from their protein sequences, but details of their arrangement in these compartments are still debated [66]. For example, it has been proposed that the enzymes are at different membrane locations throughout the Golgi, and in that configuration, they would randomly synthesize chains depending on overall luminal availability of UDP-sugars and PAPS substrates. More recently, studies with chemically modified xylosides that serve as "substitute" acceptors for CS synthesis in the Golgi [43, 269] suggest that distinct functional macromolecular assemblies of elongation and sulfation enzymes, termed "GAGOSOMES", are present. These complexes would concurrently catalyze the UDP-sugar addition and sulfate transfer to generate diverse GAG chain structures. This type of mechanism could indeed account for the differences in CS chain structures present on the CS1 and CS2 domains of aggrecan. The need for a specialized configuration of the Golgi compartment to achieve coordinated glycosylation reactions has also been suggested from genetic mutations in proteins such as COG4, CORAB and GOG8 associated with Golgi subdomains. These proteins have been shown to cause congenital disorders of glycosylation, including GAG biosynthesis, due to mis-localization of the transferase enzymes [2, 99, 167]. Topographical organization of the GAG biosynthetic enzymes is also a necessary prerequisite for targeted transport of nucleotide sugar precursors [242] for glycosylation and PAPS for sulfation [18, 57] from their production sites in the cytosol into the ER/Golgi lumen. In this regard, genetic deletion of the nucleotide sugar transporter Slc35d1 caused a skeletal defect in the knockout mice, and this was due to a sparse substitution of significantly shortened CS chains on aggrecan [98]. Other factors that could influence a functional Golgi membrane structure and luminal environment, and thereby regulate core protein glycosylation, include pH [213], ionic strength, [137] and cellular stress responses [225].

1.2.6 Alterations in CS Fine Structure by Biomechanical Stimuli – What Parts of the Post-translational Pathway Are They Targeting?

While there have been studies on the effects of growth factors (e.g. TGFβ1, IGF1) and cytokines on cartilage GAG synthesis, [161] and on CS synthesis [22, 171, 188], there have been relatively few studies to determine the effects of biomechanical stimuli on modulation of CS and KS synthesis enzymes. Cyclic compression of bovine cartilage explants in vitro resulted in the synthesis of CS chains with increased GalNAc6-sulfation and a concomitant decrease in GalNAc4-sulfation, and with fewer chains terminating with disulfated GalNAc4,6S [28, 227]. In vivo treadmill exercise in horses [28] increased CS chain size, which was accompanied by a greater proportion of un-sulfated regions in the chains, suggesting a differential effect on the supply of UDP-precursors and PAPS to the CS-synthesizing enzymes, or a selective decrease in activity of the sulfotransferases.

However, a considerable number of studies have reported structural changes in the cytoskeleton and intracellular organelles, such as mitochondria, ER/Golgi [145] and the nucleus, and in structures in response to biomechanical stimuli, including compression, hydrostatic and osmotic pressure [29, 32, 53, 56, 64, 82, 95, 123, 125, 128, 137–139, 145, 168, 169, 253]. Likewise such mechanical perturbations of the tissues or the cells is expected to modify ion channel activity, Ca2+ signaling [53, 101, 196, 299, 300] and glucose transport and utilization [138, 160, 241, 276, 286] that can affect steps in glycosylation pathways.

In summary, mechano-signal transduction, [77] which targets the aggrecan GAG substitution pathways, is likely to induce changes in the GAG precursor synthesis and/or topographical organization of the GAG synthesis enzymes,

rather than in transcriptional regulation of the GAG biosynthetic enzymes (Fig. 1.1).

1.3 Aggrecan Metabolic Turnover in the ECM of Healthy and Osteoarthritic Cartilages

The cartilage ECM composition changes in order to adapt to various postnatal stages of growth and maturation, and is also affected by arthritic diseases. The mechanisms that such metabolic turnover events have on aggrecan have been well studied. For example, Maroudas and coworkers [156, 268] measured the D/LAsp ratio and the advanced glycation end product, pentosidine, in aggrecan purified from adult human cartilages and reported a half-life of ~3 years in vivo. A different approach [91] utilized an in vitro cartilage explant culture method with medium supplemented with 35S radiolabel to tag the CS-bearing region of newly synthesized aggrecan. By quantitating both the matrix retention and release into the culture medium of newly synthesized and resident CS-core protein fragments, turnover constants and half-lives for both pools of aggrecan in vitro were determined to be between 6-20 days. This method was subsequently used by others [35] to show that the half-life of aggrecan in the ECM can be prolonged by the inclusion of serum or anabolic growth factors [35, 172] or was shortened by proinflammatory stimulators [88] in the culture medium. It is also influenced by the type of cartilage [197] or the disease state [37, 219], and can be modulated by biomechanical perturbations [58, 133, 191, 205–207, 217].

1.3.1 Enzymatic Mechanism of Aggrecanolysis

Explant culture experiments demonstrated that a cell-dependent process generates aggrecan species that can no longer bind to HA and therefore diffuse from the tissue. This in turn motivated a research area to determine the molecular mechanism for the “aggrecanolysis”.

Our understanding of “aggrecanolysis” in the human joint was clarified by detailed analysis of aggrecan intermediates in chondrocyte and cartilage culture medium [103, 222], and this was shown to occur naturally in human cartilage and synovial fluids [220] (Fig. 1.3). The most studied aspect has been the proteolysis of the interglobular domain (IGD) of aggrecan with the release of the glycosaminoglycan (GAG)-attachment regions which is destructive to the tissue biomechanical function [20, 21] as it causes loss of the CS from the cartilage ECM.

Although there had been much debate around data suggesting a role for MMP3 (Stromelysin) in aggrecanolysis, a team of scientists at the pharmaceutical company DuPont [258] purified the aggrecan degrading proteolytic enzymes from the medium of catabolically stimulated bovine cartilage explant cultures. They belonged to the “A Disintegrin and Metalloproteinase with the ThromboSpondin motifs” (ADAMTS) family of metalloproteinases. They were termed aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5).

1.3.2 Targeted Inhibition of Aggrecanolysis – A Potential Treatment for Human Osteoarthritis?

Given that aggrecan depletion of the articular cartilage is a hallmark of chronic OA and that ADAMTS5 has been proposed as the primary aggrecanase responsible for the destructive cleavages [73, 78, 246], it appeared likely that inhibitors of this enzyme would have therapeutic value as a Disease Modifying OA Drug (DMOAD).

A number of preclinical studies with in vitro explant cultures and/or animal models of OA using small molecular weight inhibitors of ADAMTS5 [25, 41, 45, 46] and catalytic-site directed neutralizing antibodies [192] showed promising results, and several of these potential therapeutics were tested in clinical trials (Table 1.4). However to-date, although showing promising DMOAD activity in pre-clinical models of OA [40, 134, 166], none were effec-

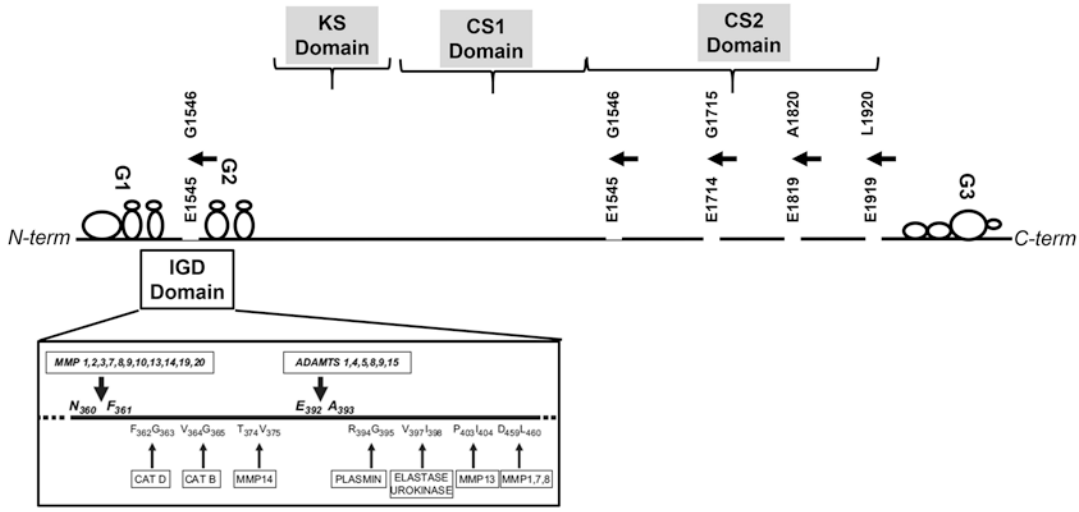

Fig. 1.3 Proteolysis sensitive sites in the human aggrecan core protein: The amino acid sequences in the sciscle bonds were either identified by protein sequencing of fragments isolated from human cartilages or synovial fluids (for MMPs, N-F and for ADAMTS, E-A, E-G and E-L) [220] or predicted from the published aggrecan core protein sequences [60]

Table 1.4 ADAMTS-5 inhibitors advancing into human clinical trials

Drug	Clinical trial ID and duration	Outcome Measures and Study Subjects	Published Data
Small molecule inhibitors			
AGG-523 Wyeth	NCT00454298 Phase I (2007–2009)	Evidence for aggrecan catabolism in urine, blood, or the knee joint Pharmacokinetics and safety profile after taking the drug either once a day or twice a day for 4 weeks. Healthy and OA patients	NO Data available
AGG-523 Wyeth	NCT00427687; Phase I (Feb 2007–June 2007)	The effect of AGG-523 on biomarkers related to osteoarthritis	NO Data available
GLPG1972 Galapagos	NCT02612246; Phase I (April 2016–July 2016)	Toxicity, pharmacokinetics, pharmacodynamics Healthy and OA patients	[25]
GLPG1972 Galapagos	NCT03595618 Phase II (August 2018–July 2020)	Reduction in cartilage loss was assessed by cartilage thickness as measured in the medial cMTFC of the target knee using qMRI. OA Patients	www.fiercebiotech.com/biotech/galapagos Shows no DMOAS activity
Antibodies			
M6495 Ablynx	NCT03583346 Phase I (August 2018–July 2019)	In participants with symptomatic knee OA to explore the safety, tolerability, immunogenicity, pharmacokinetics (PK), and pharmacodynamics (PD)	NO Data available
M6495 Ablynx	NCT03224702 Phase I	Healthy Male Subjects	NO Data available

tive in the human disease, or showed detrimental side effects and thus not approved for clinical use. For example, in human OA explants, a humanized ADAMTS-5-selective monoclonal antibody (GSK2394002) was able to decrease the levels of aggrecan fragments released. However, toxicity studies of this antibody in a primate model of OA showed impairment of cardiovascular function as a side effect, and clinical trial studies were not developed. A novel type of therapeutic anti-ADAMTS-5 antibody, the Nano-body (M6495, Ablynx) blocked OA progression in mice following destabilization of the medial meniscus (DMM) surgery and reduced circulating levels of aggrecanase-generated aggrecan fragments when administered in a primate model [26]. A different set of antibodies that inhibited either the ability of ADAMTS5 for auto-activation or its interaction with an activating factor, such as LRP1, have also been shown to protect against aggrecanolysis in vitro [223, 224]. However, no information is available if they were investigated for their clinical therapeutic usefulness.

In summary, future plans for the generation of aggrecanase inhibitors as clinically sound therapeutics for targeted mitigation of aggrecan depletion from the cartilage ECM during OA pathogenesis may remain impeded by the findings that these enzymes have multi-tissue and organ distributions and functions. For example ADAMTS5 is essential for dermal wound healing [266], maintenance of tendon fibrillar structure/function [275], regulation of metabolic health by adipose tissue [17], and cardiovascular homeostasis [16]. An alternative future approach to restoring the aggrecan-dependent physiochemical and biomechanical properties of the cartilage matrix may require the cartilage-targeted delivery of engineered cleavage-resistant aggrecan-or GAG-mimetics, singly or in molecular complexes with other components. Such an approach could develop from technological advances made to-date in chemo-enzymatic synthesis of functional GAG structures and domains [175, 240].

1.4 Hyaluronan Metabolism and Its Relevance to Cartilage Structure and Function

Hyaluronan is a high-molecular weight polysaccharide composed of repeating disaccharide units, (→4) β-GlcA (1→3) βGlcNAc (1→) with a wide range of structural and metabolic functions in all tissues and body fluids [89]. These functions include lubrication, water homeostasis, macromolecular filtering, interactions with "hyaladherins" in matrix organization [49, 158, 274, 303] and regulation of cellular activities during development and in a range of pathologies [76, 92, 130, 194, 257]. This section provides a brief summary of the extensive research into the role of HA in cartilage structure/function and follow with highlights of recent advances in HA metabolism that could be incorporated into studying the cell biological responses of tissues under mechanical perturbations.

1.4.1 Hyaluronan in Cartilage Matrix Structure and Articular Joint Mechanics

The role of HA in cartilage has largely been considered in the light of its physical properties, namely for organizing aggrecan throughout the extracellular cartilage matrix A first report of a specific interaction of aggrecan with HA was reported by Hardingham and Muir [86, 87, 260], followed by more detailed analyses of the role of HA chemistry [90] and the role of the link glycoproteins in stabilization of the protein carbohydrate interactions [23, 67, 182, 252]. The biochemical analyses was later confirmed by electron microscopic methodology to visualize the structural arrangement of aggrecan monomers [96, 215] and link proteins [30, 31, 173] along the extended HA polymer backbone. In vitro cell biological studies with rat chondrosarcoma cells, and with pig and rabbit articular chondrocytes, confirmed that the ternary com-

plex between aggrecan, link protein and hyaluronan was formed extracellularly, soon after secretion of the glycosylated proteins from the cell [120, 121, 198, 209, 216].

A different protein-HA modification, first discovered in the cumulus oophorus extracellular matrix [74] has also been identified in the extracellular matrix of OA cartilage [296]. These macromolecular HA complexes are formed in the extracellular matrix by covalent transfer of heavy chains (HCs) from inter-alpha-inhibitor (ITI) to HA. ITI is a modified CS proteoglycan with a core protein, bikunin that has 1, 2 or 3 HCs attached by an ester linkage between an aspartate in the HC and the 6-OH of a GalNAc in the CS chain [150]. The HC is transferred to the 6-OH on GlcNAc in HA [301] by tumor necrosis factor-induced protein-6 (TSG-6) [48, 176]. Subsequent investigations have identified the formation of such HC-HA matrices as part of a cellular response in tissue inflammations in a wide range of chronic diseases [136, 274], including asthma [250] Crohn's disease [195], diabetic nephropathy [141], and degenerative suspensory ligament desmitis [202]. In both, OA and RA, HA-HC complexes are abundantly present in synovial fluid aspirates from patients [116, 229, 293, 296] and in animal models [68, 135] likely having been shed into the fluid after formation in inflamed synovium and/or degenerated cartilage.

In addition to the role of HA in organization of tissue and cell-specific extracellular matrices, it generates the viscoelastic properties of synovial fluid [185, 251], and in cooperation with the mucin-like molecule, PRG4 (aka Superficial Zone Protein or Lubricin), it provides boundary lubrication of the articular cartilage surfaces in diarthrodial joints [230]. Notably, in both OA and RA, decreased size and increased polydispersity of molecular the weight distribution of HA polymers in synovial fluid have been reported [12, 13] in keeping with the proposed impaired cartilage boundary lubrication in degenerative joint diseases [24]. Such observations led to the wide clinical use of intra-articular injections of high molecular weight HA as potential therapeutic 'viscosupplementation' for arthritic joints [4, 10, 11, 211].

1.4.2 Engagement of Hyaluronan Receptors Modulates Cell Responses

The studies of HA receptors, CD44, RHAMM, LYVE, Layilin and Stabilin2 and their downstream effects on cellular functions have been extensively investigated, particularly in the areas of development, cancer and respiratory diseases, as well as neuro- and vascular pathologies. A number of comprehensive recent reviews on this topic are available [76, 111, 131, 146, 162, 193, 263, 281]. Several of these receptors, in particular CD44, have also been shown to be active in cartilage matrix development and inflammatory pathologies, and those reports are summarized in Table 1.5. In the context of biomechanical effects

Table 1.5 Reported in vivo and in vitro functions of HA receptors in mechanosentive joint tissues

Receptor	Cartilage/Synovium	Bone
CD44	Immobilization of pericellular HA [129]; Cell adhesion [132, 147]; Endocytosis of HA [3]; Modulation of BMP7 signaling [151]	Unloading and inflammation induced bone loss [94, 143] Osteoclast multinucleation [51]
RHAMM	Localized in epiphyseal cartilage, articular fibrocartilage [62]; Modulation of expression of transcription factor Nrf2 in chondrocytes [189] Decreased IL6 and IL8 production, decreased migration of synoviocytes [287]	Differentiation of osteoblasts [93]
Layilin	Modulation of cytokine expression [8] Inhibition of IL-1β-induced MMP-1 and MMP-13 production in synoviocytes [177]	**No reports**
LYVE	Synovial biomarker for joint inflammation [102]; Lymphatic and blood **ve**ssel ingrowth in endplate cartilage [218] Increased lymphatics in OA and RA synovium [289]	Deficient lymphatics in peri-implant membrane [65]

on receptor HA interactions it is notable that ligand responses to tensile or flow stresses have been reported [14, 15, 184, 208], which would imply that application of physiological forces, such as tensile stress, sheer stress and fluid flow can affect receptor-HA interactions. This would provide an important function of these cell/matrix interactions as force sensing mechanisms [71, 148].

1.5 HA Metabolism Pathways Support Cell Survival

The biophysical, structural and cell biological roles of HA polymers reviewed above should be viewed in relation to their biosynthesis and degradation pathways. Over the past 5 decades many laboratories contributed research data that have built a comprehensive picture of these pathways (see Fig. 1.4).

1.5.1 Enzymatic Pathways in HA Synthesis and Catabolism

The first insights into the mechanism of HA synthesis were reported in 1959, using Streptococcus membranes [154] that contained an enzyme activity (HA synthase (HAS)), which uses GlcA-UDP and GlcNAc-UDP as substrates to polymerize HA chains, and its gene was cloned in 1993 [52]. This was followed by identification of mammalian HAS genes (HAS1, HAS2 and HAS3) from a number of laboratories (reviewed in [282, 284]). They are transmembrane proteins, and have similar domain organizations that allows the direct translocation of the HA polymer into the extracellular space during HAS-catalyzed synthesis [153, 280]. Rates of polymer synthesis and size of the extruded HA chain are dependent on expression, translation and plasma membrane targeting of the enzyme proteins [255] as well as

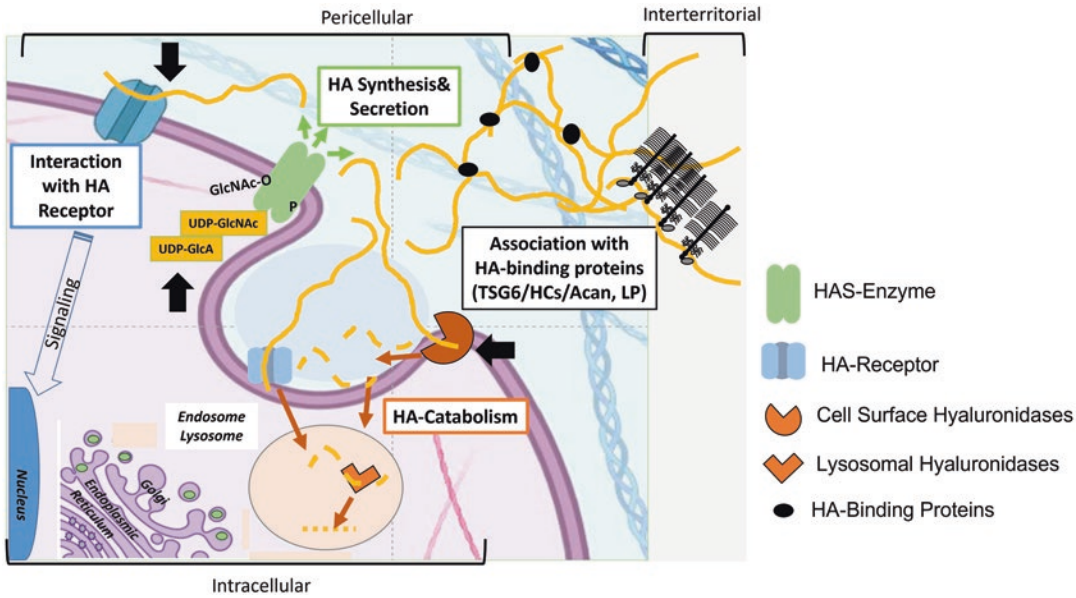

Fig. 1.4 Schematic illustration of coordination of HA synthesis, catabolism and HA-protein interactions: HA-Synthesis steps include HAS1, 2 or 3 protein transcription, modification and translocation to the plasma membrane, polymerization of HA chains using cytosolic UDP-GlcA and UDP-GlcNAc precursors and extrusion into the extracellular space. Cell signaling can be induced by HA/cell surface receptor interactions (CD44, RHAMM, Layilin). Interaction of HA with binding proteins (Acan, LP, TSG6, HCs) in the pericellular and interterritorial matrix generate specialized macromolecular complexes. HA-catabolism is mediated either by receptor mediated internalization (via LYVE-1 or Stabilin-2) of high molecular weight polymer or of low molecular weight fragments generated by cell surface hyaluronidases (TMEM2 or CEMIP) and completed in the lysosomal compartment by resident hyaluronidases (HYAL1, HYAL2 or HYAL3)

on the supply of the UDP-sugar precursors from the cytoplasm [92, 112]. A detailed study of HAS2 has revealed additional levels of post-translational control, including phosphorylation, [270], O-GlcNAcylation, [112], ubiquitination and dimerization [114]. Furthermore, the establishment of HAS knock out mouse strains provided important insights into the distinct roles of the three HAS proteins in development, growth and pathologies (summarized in Table 1.6).

In addition to the biosynthetic pathways, the degradative mechanisms for HA in tissues is also becoming more clearly defined. The existence of lysosomal hyaluronidases has long been established [110, 259], and their involvement following receptor mediated endocytosis via CD44 [47, 85], LYVE-1 [204] and HARE (Stabilin 1) [283]. However, extracellular hyaluronidase activities remained elusive until the identification of two extracellular hyaluronidase activities: (1) TMEM2, a type II transmembrane protein with hyaluronidase activity at neutral pH, [105, 256, 291] is expressed widely in adult mouse tissues, including vascular and lymphatic endothelial cells and liver, the major sites of HA clearance; and (2) KIAA1199 (CEMIP) [294, 295]. CEMIP was initially described as having a pivotal role in cancer cells, aiding their migration during tissue invasion and metasis [72, 262]. However, a number of recent reports have demonstrated its involvement in both cartilage pathologies [54, 59, 235, 299, 300] and osteoblast differentiation [39] making this an interesting candidate gene and protein to examine in relation to biomechanical stimulants imposed on cartilage and bone tissues (see Fig. 1.4).

Table 1.6 Genetic deletion of HA synthases and hyaluronidases in mice

Gene/protein	Phenotype knock-out mouse strains
Has1 (hyaluronan synthase 1)	Defective formation of retrocanal Bursa [237] Increased Synovial Fibrosis, Osteopenia [38]
Has2 (hyaluronan synthase 2)[a]	Impaired skeletal development [159, 170] Increased airway hypersensity in asthma [233]
Has3 (hyaluronan synthase 3)	Altered neuronal activity [6] Decreased neointimal hyperplasia [118, 231] Increased tumor cell invasion in human mammary parenchymal tissues [140]
Hyal1 (hyaluronidase 1)[b]	Accelerated thinning of knee joint cartilage in aging Prolonged fertility [157]
Hyal2 (hyaluronidase 2)	Severe cardiopulmonary dysfunction, Anemia, Mild craniofacial abnormalities [42]
Hyal3 (hyaluronidase 2)	No detectable phenotype [9]
Tmem2[c] (Transmembrane protein 2; aka CEMIP2)	Increased levels of circulating HA, active on the surface of endothelial cells in the lymph nodes and liver [256]
Cemip (aka KIAA1199)	Impaired learning and memory ability due to decreased dendritic spine density in dentate gyrus granule cell [297]

[a]Conditional and Heterozygous Knockout Strains only; complete Knockout is embryonically lethal due to failure of heart development [34]
[b]Human Mucopolysaccharidosis Type IX is due to a mutation in the HYAL1 gene
[c]Conditional Knockout Strains

1.5.2 Synergy Between Glucose Metabolism and HA Synthesis Adjusts the Cellular Energy Status

More recent studies on HA metabolism in cancer biology and diabetes have clearly demonstrated that biosynthesis of the HA is closely linked to intracellular glucose metabolism. This is through both aerobic and anaerobic glycolysis for energy production [265], and by the generation of the two sugar nucleotides, UDP-GlcNAc and UDP-GlcA. Together these sugar nucleotides regulate HA production by modification of both the biosynthetic activity [272, 304] and the half-lives of the membrane-associated HAS enzymes [271].

Biosynthesis of the two nucleotide precursors takes place in the cytoplasm (Fig. 1.5) and is driven by the availability of intracellular glucose taken up by the cell from the interstitial fluid by glucose transporters and its subsequent conversion to Glc6P [36]. UDP-GlcNAc is then synthesized

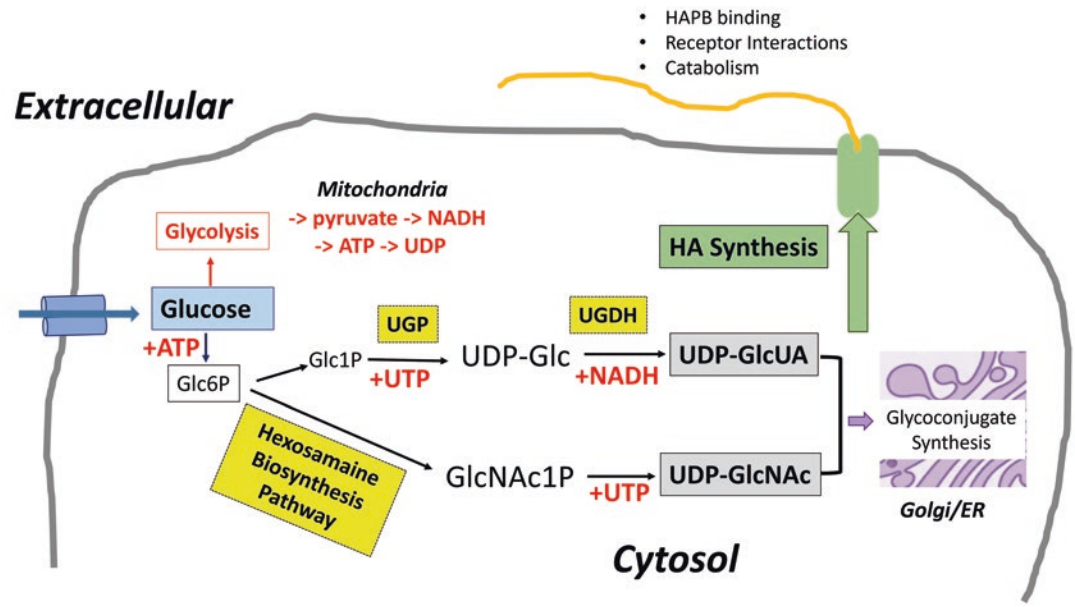

Fig. 1.5 Schematic Illustration of Integration of Glucose Metabolism for Cytosolic Production of HA Biosynthesis Precursors UDP-GlcNAc and UDP-GlcUA: Extracellular glucose is transported into the cytoplasm by specific glucose transporters, where it is shunted for energy production via glycolysis and for production of the HA synthesis precursors UDP-GlcNAc and UDP-GlcUA via the hexosamine biosynthetic pathway or by UDP-glucose pyrophosphorylase/UDP-glucose dehydrogenase, respectively. Potential regulatory sites for mechanical stimuli of cells/tissues are indicated by bold black arrows. It should be noted that HA synthases have 'direct' access to cytosolic UDP-precursors, whereas UDP precursors for the chondroitin and keratan polymerases, or for other enzymes of glycol-conjugate synthesis, require an additional translocation/transport step into the ER/Golgi compartments

via the hexosamine biosynthetic pathway [187], that also engages products from amino acid metabolism (glutamine) and lipid metabolism (Acetyl-CoA). UDP-GlcA biosynthesis on the other hand, depends on the activity of two enzymes, UDP-Glucose pyrophosphorylase (UPP), which uses glucose-1-phosphate (Glc1P) and UTP to generate UDP-Glc for conversion to UDP-GlcUA by UDP-Glucose dehydrogenase (UGDH) [244, 304]. Both enzymes show a wide tissue distribution, including cartilages [44, 152].

To date, the mechanistic linkage of glucose metabolism and HA synthesis has not been studied in detail in the context of cartilage during growth, maturation and pathologies, with only one recent review pointing to its importance in the developmental biology of the tissue [100]. An interest in the importance of the HBP in OA pathology was initiated by the observations that high concentrations of extracellular glucosamine or mannosamine could inhibit in vitro cytokine-induced aggrecan degradation by ADAMTS proteinases [190, 221] and inhibit disease progression in animal models of OA [183, 273]. Clinical use of oral dosages of glucosamine as a potential DMOAD [19, 70, 109, 165, 212] is still debated.

1.5.3 Are Biophysical Stressors Important in Regulation of HA Metabolism by Chondrocytes?

The subject of biomechanical effects on HA metabolism has been most broadly studied in endothelial cells and their response to sheer stresses generated by blood flow [81, 155, 277], as well as in epithelial cells in the alveolar lining [97]. Other mechanical perturbances, such as cyclic mechanical stretch or strain, shear stress, surface motion or mechanical injury [63, 119, 138, 142, 210, 254, 298] imposed on connective tissue cells, including fibrochondrocytes and articular

chondrocytes, have also been shown to modulate HA production. The later studies have not provided any information on potential transduction pathways for stimulated HA production, but likely mechanisms could come from the newly emerging databases on cartilage "metabolomics' [5, 50, 232, 236, 243]. Key regulatory points would include glucose transport [168, 169, 241], subsequent Glc6P shunting to aerobic [113] or anaerobic glycolysis [292, 302] for energy production, and/or synthesis of UDP-GlcNAc and UDP-GlcUA to regulate HAS activities. Given the critical structural and cell regulatory roles of HA reviewed above, a more detailed understanding of HA metabolism and its response under biomechanical perturbation of tissues and cells would provide novel opportunities to uncover treatment of cartilage pathologies [261], as well as optimization of procedures for the production of tissue engineered cartilages [160, 245].

1.6 Conclusion

Despite the extensive knowledge base in cartilage extracellular matrix structure and metabolism in health and diseases, there remain multiple opportunities to apply 'big data' generation and bioinformatics mining approaches to gain further insights to the feed-forward and feed-back mechanisms between genes, their products and cellular pathways. These goals could be achieved by applying such approaches to examine engineered tissues, animal models and clinical biorepositories.

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