Part I
Matrix Biology

CHAPTER 1

Matrix Biology: Extracellular Matrix – Building Function Through Complexity

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1.1 INTRODUCTION

1.1.1 The ECM

The extracellular matrix (ECM) is the extracellular component of a multicellular organism or tissue that provides structural and biochemical support to the surrounding cells. Characteristic of ECM is a complex interaction of specific large and small molecules that function as a composite structure: these structures can vary in different parts of the extracellular environment from a pericellular localization (concentrated around the cell) to interterritorial, making up the bulk of the ECM. These complex networks confer the functions of the ECM that are tissue specific; they are also dynamic, changing over time and developmental stage as well as in a response to injury or disease. ECM

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is also a storehouse for molecules that can be released at later times, including growth factors that bind to the charged glysocaminoglycan chains of proteoglycans such as fibroblast growth factors (FGFs) and growth factors that bind to protein domains, like bone morphogenetic proteins (BMPs) and transforming growth factor beta superfamily members (TGFβs). The resident cells take their cues from their developmental program or information from cell–matrix or cell–cell interactions and synthesize appropriate ECM. Each tissue is exquisitely designed for its particular function, providing structural support and functional information that feeds back to the ECM-producing cells. For example: skin requires a barrier function, elasticity and wound healing ability; cartilage requires the ability to compress, functioning as a shock absorber; muscle requires a high capacity for work; and tendons require the ability to bend, pull and tighten. The ECM, made up of specific mixtures of collagens, proteoglycans, glycoproteins and other secreted molecules, performs all of these functions. The complexity of ECM structure and function provide a distinct challenge to tissue engineers. Figure 1.1 shows a freeze-etch electron micrograph of a chondrocyte in ear cartilage surrounded by ECM[1] while Figure 1.2 illustrates the types of molecules present in the cartilage ECM.[2]

Often the major structural component of ECM is a genetically distinct collagen, such as in skin and bone (type I collagen), tendon

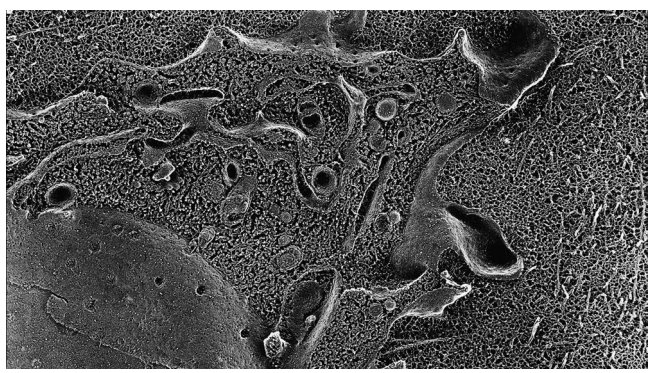


Figure 1.1 Chondrocyte with ECM. Quick-frozen, non-fixed, freeze-etched electron micrograph of a 150 day gestation fetal bovine ear chondrocyte surrounded by ECM. The cell is in the lower left area of the photograph and the matrix is in the upper right. Notice the very close association of the matrix with the cell. The plasma membrane of the cell is highly convoluted with numerous projections and infoldings. Nuclear pores are evident on the nuclear membrane, which is continuous with the cytoplasmic membrane system. The extracellular matrix consists of a finely woven meshwork that adjoins the cell membrane and is continuous throughout the extracellular space.[9]

(type III collagen), cartilage (type II collagen), cornea (type V collagen) and basement membrane (type IV collagen) often in a combination with other functional components, *e.g.* elastin for skin, proteoglycan aggrecan for cartilage, or calcium phosphate mineral for bone. Often tissues with highly functional ECM have a low cell to matrix ratio, where the cells in the tissue lay down a predictable amount of matrix during differentiation, development and growth (such as cartilage, bone, tendon). Functionally the centerpiece of connective tissues, ECM can resist compression (cartilage), make strong attachments (tendon, ligament), provide structure to the organism (bone), direct differentiation and development (mesenchyme) and guide nerve and blood vessels. Components of the ECM often assemble into large macromolecular functional units with interactions among molecules of the same or different types.

How can ECM perform so many different functions? Besides the major macromolecules the ECM harbors smaller amounts of

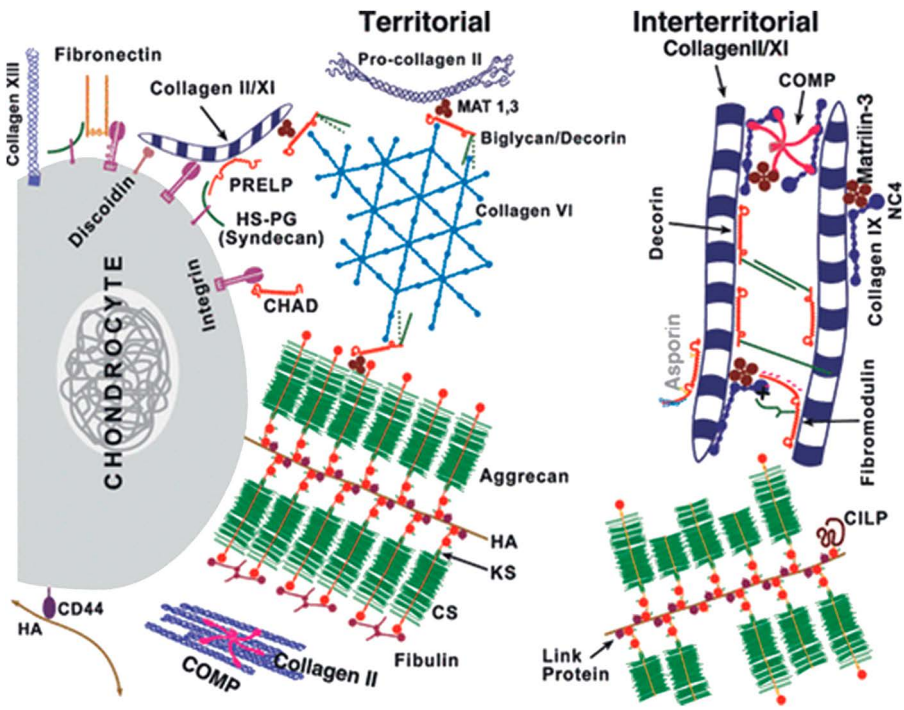


Figure 1.2 Schematic representation of cartilage ECM showing molecular constituents in cartilage and their arrangements into large multimolecular assemblies. The different compositions and organizations of macromolecules is seen at the cell surface with the number of receptors interacting with specific matrix molecules, at the interterritorial matrix closer to the cells, and the interterritorial matrix at a distance.[2]

functional molecules critical to structural functions, signaling functions, and specific degradation mechanisms. The ECM is also the home of many factors that are stored for later use, like MMPs (mobilized during injury) and growth factors (mobilized during development and regeneration).

1.1.2 Water

Water is a critical component of the ECM. The macromolecular composition of the matrix determines the matrix hydration that, in turn, determines tissue volume, creates space for molecular transport and for dynamic organization and offers compressive resistance. In the ECM, water is generally extrafibrillar (in relation to collagen fibers) and its distribution is specifically determined by the concentration of dissolved and retained proteoglycans and glycoproteins (>2 mg ml^{-1}).[3] The hydrodynamic processes controlling the water content in the ECM are those of osmosis, filtration, swelling and diffusion. In many cases, the macromolecular components influencing these hydrodynamic processes are nonideal. The nonideality (which yields parameters that vary nonlinearly with concentration) influences osmotic pressure through excluded volume polymer interactions and, for charged polymers, through the influence of active counterions on the ambient simple electrolyte concentration. Nonideal effects are manifested in dynamic parameters as well, particularly the hydrodynamic frictional terms that describe the viscous dissipation of water over the surface of the polymer chain.

The ECM functions to control many critical parameters of tissue homeostasis and response such as proliferation, apoptosis, development and morphogenesis. In this chapter, I will describe many (but not all) of the predominant ECM components and special functions required by different matrices.

1.2 MAJOR STRUCTURAL AND FUNCTIONAL COMPONENTS OF ECM

1.2.1 Collagens

The most abundant protein in all multicellular organisms is collagen and all mature collagen resides in the ECM. There are now known to be 28 different collagen types made up of at least 46 genetically distinct polypeptide chains; many other proteins contain collagenous domains. The most abundant collagens are type I (bone, skin), type II (cartilage and meniscus), type III (tendon, blood vessels), and

type IV (basement membranes). Table 1.1 lists the different collagen types, their component polypeptide chains (gene products) and tissue sources.[4] The most abundant collagens (fibrillar collagen types I, II and III) are approximately 3000 nm in length and made up of uninterrupted Gly–X–Y repeats where X and Y are often proline and

Table 1.1 Vertebrate collagens.[a]

Type	Class	Composition	Distribution
I	Fibrillar	$\alpha1[I]_2\alpha2[I]$	Abundant and widespread: dermis, bone, tendon, ligament
II	Fibrillar	$\alpha1[II]_3$	Cartilage, meniscus, vitreous
III	Fibrillar	$\alpha1[III]_3$	Skin, blood vessels, intestine
IV	Network	$\alpha1[IV]_2\alpha2[IV]$ $\alpha3[IV]\alpha4[IV]\alpha5[IV]$ $\alpha5[IV]_2\alpha6[IV]$	Basement membranes
V	Fibrillar	$\alpha1[V]_3$ $\alpha1[V]_2\alpha2[V]$ $\alpha1[V]\alpha2[V]\alpha3[V]$	Widespread: bone, dermis, cornea, placenta
VI	Network	$\alpha1[VI]\alpha2[VI]$ $\alpha3[VI]$ $\alpha1[VI]\alpha2[VI]$ $\alpha4[VI]$	Widespread: bone, cartilage, cornea, dermis
VII	Anchoring fibrils	$\alpha1[VII]_2\alpha2[VII]$	Dermis, bladder
VIII	Network	$\alpha1[VIII]_3$ $\alpha2[VIII]_3$ $\alpha1[VIII]_2\alpha2[VIII]$	Widespread: dermis, brain, heart, kidney, cartilage
IX	FACIT[b]	$\alpha1[IX]\alpha2[IX]\alpha3[IX]$	Cartilage, cornea, vitreous
X	Network	$\alpha1[X]_3$	Cartilage hypertrophic zone
XI	Fibrillar	$\alpha1[XI]\alpha2[XI]\alpha3[XI]$	Cartilage, intervertebral disc
XII	FACIT	$\alpha1[XII]_3$	Dermis, tendon
XIII	MACIT	—	Endothelial cells, dermis, eye, heart
XIV	FACIT	$\alpha1[XIV]_3$	Widespread: bone, dermis, cartilage
XV	MULTIPLEXIN	—	Capillaries, testis, kidney, heart
XVI	FACIT	—	Dermis, kidney
XVII	MACIT	$\alpha1[XVII]_3$	Hemidesmosomes in epithelia
XVIII	MULTIPLEXIN	—	Basement membrane, liver
XIX	FACIT	—	Basement membrane
XX	FACIT	—	Cornea (chick)
XXI	FACIT	—	Stomach, kidney
XXII	FACIT	—	Tissue junctions
XXIII	MACIT	—	Heart, retina
XXIV	Fibrillar	—	Bone, cornea
XXV	MACIT	—	Brain, heart, testis
XXVI	FACIT	—	Testis, ovary
XXVII	Fibrillar	—	Cartilage
XXVIII	—	—	Dermis, sciatic nerve

[a]Modified from Shoulders and Raines, 2009.
[b]Abbreviations: FACIT, fibril-associated collagen with interrupted triple helices; MACIT, membrane-associated collagen with interrupted triple helices; MULTIPLEXIN, multiple triple-helix domains and interruptions.

hydroxy proline. In some collagens, this Gly–X–Y sequence is interrupted by globular protein sequence. Type IV collagen is about the same length, but contains many interruptions in the Gly–X–Y sequence. Three polypeptide chains of a single gene product, or multiple gene products, intertwine to form the collagen molecule. The characteristic amino acids give collagen its unique helical structure and the defining structural motif in which each of the three parallel polypeptide strands form a left-handed, polyproline II-type (PPII) helical conformation; the three chains then supercoil about each other with a one-residue stagger to form a right-handed triple helix. The individual collagenous proteins are composed of several functional domains involved in biosynthesis, fibrillogenesis, fiber structure cross-linking, cell or molecular interactions, and degradation.[5] The categories of collagen include the classical fibrillar and network-forming collagens, the fibril-associated collagens with interrupted triple helices (FACITs), membrane-associated collagens with interrupted triple helices (MACITs), and multiple triple-helix domains and interruptions (MULTIPLEXINs). In the ECM, the collagen fiber can be made up of more than one collagen type and even contain non-collagenous components.

Collagens are encoded for by large genes made up of many exons that are the functional units of the protein and regulatory apparatus. Evolutionarily, collagen arose from a single 54 base pair exon encoding 18 amino acids forming 6 Gly–X–Y triplets.[6] This exon was duplicated many times to form the fibrillar collagens and also incorporated into the other collagens. Various globular domains were added to the gene to accommodate various aspects of secretion, molecular interactions and degradation. Promoter and other regulatory elements allow specific genes to be expressed in the appropriate tissues. The study of gene structure of collagens is an intriguing example of evolution of very specific protein functions and interactions varying expression by means of the choice of gene regulation, alternative splicing and half life of the mRNA.

1.2.2 Large Chondroitin Sulfate Proteoglycans

Proteoglycans are large ECM components that are made up of a protein backbone and attached glycosaminoglycan chains (sulfated polysaccharides). The major aggregating proteoglycan of cartilage, aggrecan, has been studied as the paradigm[7] and functions to provide compressibility to cartilage: smaller amounts are found in meniscus

and tendon. Aggrecan contains numerous chondroitin sulfate and keratan sulfate side chains that are distributed in the C-terminal two-thirds of the core protein, while the N-terminal one-third of the molecule carries few glycosaminoglycan chains but is relatively rich in N-linked carbohydrate, most of the cysteine residues of the protein, and behaves like a globular protein (Mr = 60 000) when isolated after proteolytic cleavage. This globular domain possesses affinity for hyaluronic acid and confers on the intact monomer the property of assembling into aggregates of many monomers bound to a central hyaluronic acid strand. This aggregate is stabilized by complementary binding activity of link protein, a Mr = 45 000 glycoprotein that also binds hyaluronic acid, having mutual affinity for the proteoglycan hyaluronic acid-binding region of aggrecan. Thus the aggrecan core protein has a molecular weight of Mr > 300 000, with over 100 glycosaminoglycan side chains containing over 100 carbohydrate residues. This "monomer" attaches *via* the link protein to a strand of hyaluronic acid, making the entire complex over 1 million kD. Large aggregating proteoglycans similar to that of cartilage have been described in several other tissues, notably tendon, bone, sclera and lung, as well as aorta smooth muscle cells, skin fibroblasts and glial cells.[8]

The core protein of aggrecan is composed of three globular domains (G1, G2 and G3) with one inter-globular domain (IGD) linking G1 and G2, and two exons for keratan sulfate (KS) chain attachment (KS domain) and for chondroitin sulfate (CS) chain attachment (CS domain) situated between G2 and G3. Attachment of these GAG chains occurs on the serine of a serine–glycine dipeptide sequence present in these regions, and one molecule of aggrecan can contain up to 100 CS chains, 30 KS chains and many O- and N-linked oligosaccharides.[9] Each CS and KS chain begins in the endoplasmic reticulum (ER) with the attachment of a xylosyl residue to a designated serine: the chains are elongated into specific glycosaminoglycan chains in the Golgi where specific glucosyl transferases, sulfatases and epimerases are resident. The G1 domain comprises the N-terminus of the core protein and has the same structural motifs as the link protein. The G3 domain, making up the C-terminus, is composed of alternatively spliced epidermal growth factor-like domains, a carbohydrate recognition domain, a complement-binding-protein-like domain and a short tail.[10,11] Other large proteoglycans in this class are versican, neurocan and brevican: each has a similar structure, but less carbohydrate than aggrecan.[9]

1.2.3 Small Proteoglycans

Smaller proteoglycans also exist in the ECM of almost all connective tissues and most belong either to the small leucine-rich proteoglycan (SLRP) family with 1-3 chondroitin or dermatan sulfate side chains, or the syndecans with a heparin sulfate side chain. The SLRPs function in hydrodynamic and structural roles, organization of the ECM, fibrillogenesis and signal transduction, particularly in development, pathophysiology and tumor growth. The members of this group are differentially expressed at the protein level in many different tissues and are highly interactive (see Table 1.2): the composition or substitution of the carbohydrate also varies, leading to specific binding affinities.[12] At the protein level, SLRPs have a variable number of tandem leucine-rich repeats comprising the major control domain. Each leucine-rich repeat has a conserved hallmark motif: LSSLxLSSNxL, where L is leucine (or isoleucine, valine or other hydrophobic amino acids), and x indicates any amino acid. They are divided into 5 subclasses encoded by 18 genes located over 7 chromosomes. Clustered genes can be regulated together during development: for example, keratocan that is downstream of lumican on chromosome 12,

Table 1.2 Interactions of SLRPs with other matrix molecules.[a]

SLRPs	Matrix assembly
Class I	
Decorin	Collagen I; collagen II and III; collagen V; collagen VI; collagen XII; collagen XIV; fibronectin; thromobospondin-1; microfibril-associated glycoprotein-1 and fibrillin-1; tenascin-X
Biglycan	Collagen I; collagen II; collagen III; collagen VI; collagen IX and biglycan; collagen II and VI complex; tropoelastin and microfibril-associated gylcoproterin-1
Asporin	Collagen I
Class II	
Fibromodulin	Collagen I; collagen II; collagen VI; collagen IX; collagen XII
Lumican	Collagen I; aggrecan; β1 integrin; β2 integrin; α2β1 integrin
Osteoadherin	αvβ3 integrin; noncollagenous domain 4 of collagen IX
PREPL	Perlecan and collagen
Class III	
Osteoglycin/mimecan	Collagen I
Opticin	Heparan and chondroitin sulfate proteoglycans, collagen
Class IV	
Chondroadherin	A2β1 integrin; collagen II
Class V	
Podocan	Collagen I

[a]From Chen and Birk, 2013.

is regulated by lumican.[13] The expression of SLRPs and cytokines are regulated bi-directionally through a common regulatory framework,[14] providing feedback mechanisms regulating matrix assembly and remodeling. The GAGs of SLRPs are differentially processed in development and aging, and are variable with regard to size, number, sulfation and epimerization in various tissues. For instance, lumican is predominantly a highly sulfated proteoglycan that is present in the cornea but a glycoprotein in other tissues.[15] Variations in glycosylation (both GAG and N-linked) modify the binding affinities of SLRPs at different developmental stages and in disease. For an excellent review of the functions of small proteoglycans, please see Chen (2013).[16]

The five classes of SLRPs (http://www.uniprot.org/uniprot) are: asporin, biglycan, decorin and extracellular matrix protein 2 (class I); fibromodulin, keratocan, lumican, osteomodulin and prolargin (class II); chondroadherin, nyctalopin (class IV); podocan and podocan-like protein 1 (class V). In addition to functions in collagen fibrillogenesis, these proteoglycans affect intracellular phosphorylation, a major conduit of information for cellular responses, and modulate distinct pathways, including those driven by BMP/TGF superfamily members, receptor tyrosine kinases such as ErbB family members, and IGFI receptor, and toll-like receptors.[17]

Collagen fibrillogenesis is tightly regulated by other collagens and by SLRPs to generate tissue-specific structures and therefore tissue-specific functions. In the corneal stroma, homogenous small-diameter fibrils, which are regularly packed and arranged as orthogonal lamellae, are required for corneal transparency. Lumican-deficient mice exhibit progressive corneal opacity with age. This is associated with irregularly packed, large-diameter collagen fibrils with irregular, cauliflower-like contours in the posterior stroma. The altered fibril characteristics are consistent with dysfunctional regulation of lateral fibril growth steps. The requirement of a homogeneous population of small-diameter fibrils necessary for transparency is inconsistent with lateral fibril growth in the corneal stroma. Mice that are deficient in decorin or biglycan have only a mild phenotype. However, compound mutant mice that are deficient for both decorin and biglycan demonstrate a severe phenotype with increased numbers of large-diameter fibrils and other irregularities. Fibromodulin, which is expressed during a very narrow window in development of the corneal stroma, is involved in regulation of corneal postnatal development.[18] Indeed, high degree myopia is associated with intronic variations and single-nucleotide polymorphisms in fibromodulin, proline/arginine-rich

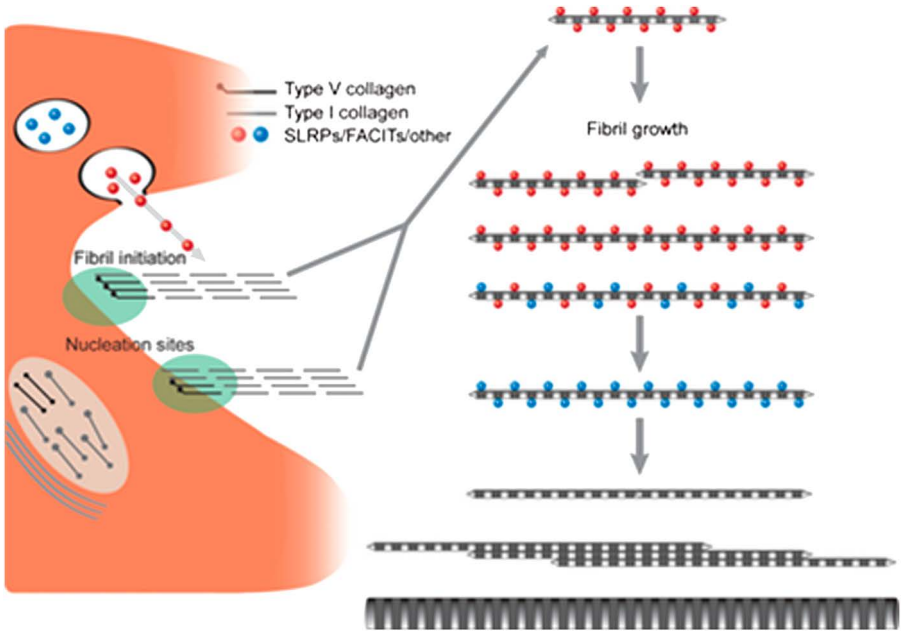


Figure 1.3 Model illustrating the involvement of SLRPs in the regulation of linear and lateral fibril growth. Collagen fibrillogenesis is a multiple-step process that is tightly regulated by the interaction of various molecules. The initial step involves heterotypic collagen I/V nucleation at the cell surface, then SLRPs bind to the protofibril surface, regulating the linear growth and lateral growth of protofibrils to mature collagen fibrils. Deficiency of SLRPs leads to dysfunctional linear and lateral fusion, with alterations in fibril structure and function.[16]

and leucine-rich repeat protein (PRELP) and opticin genes. Figure 1.3 shows the roles of small proteoglycans in collagen fibrillogenesis.

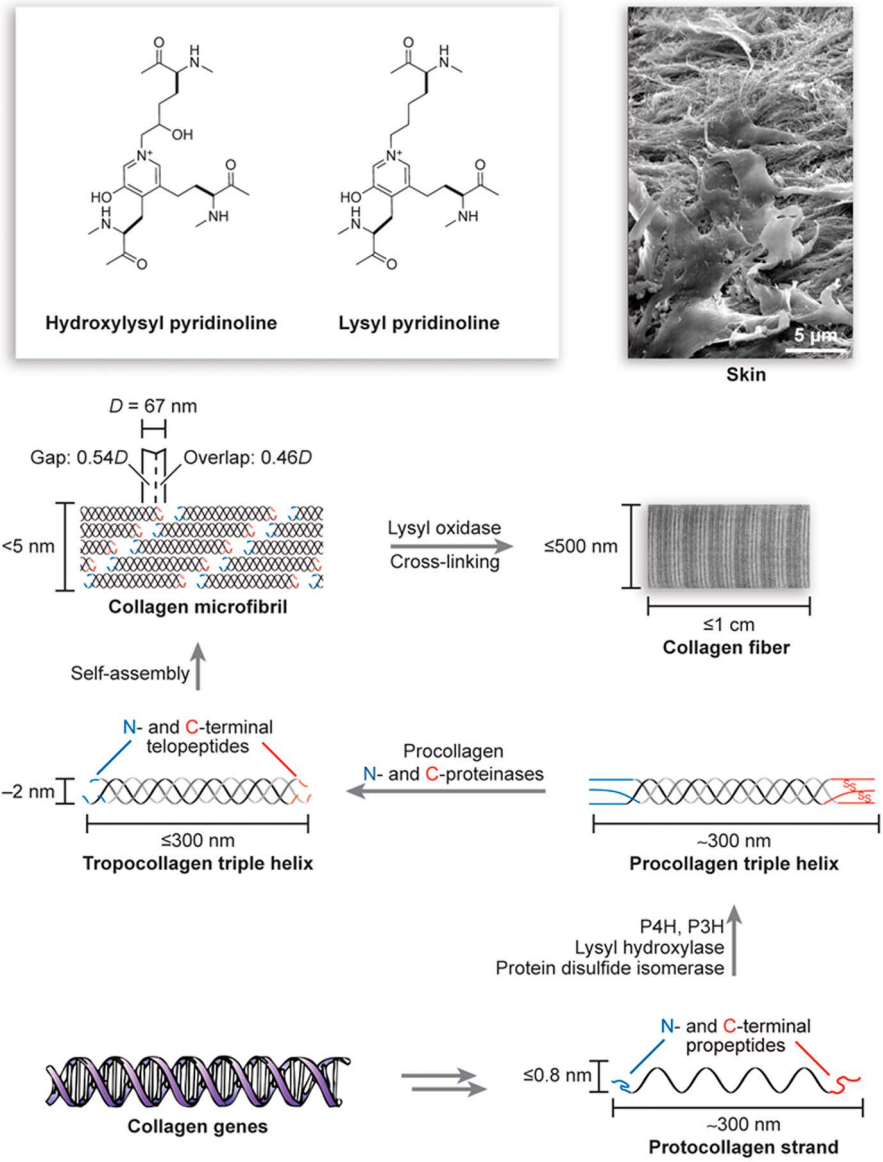
1.2.4 Biosynthesis of ECM Macromolecules

The biosynthesis of ECM components is complex due to the requirement for the extensive post-translational modifications that are vital components of almost all ECM molecules. Every ECM molecule is produced in the cell's secretory apparatus *via* the signal peptide where the proteins are transported from the cytoplasmic translational apparatus into the lumen of the ER during synthesis. The protein then undergoes specific post-translational modifications in a site-specific manner; first in the ER, then in the Golgi apparatus. The protein is exposed to the post-translational machinery that lies entirely within the lumen of the ER, where the initial direct modifications of the protein core are made as well as any N-linked oligosaccharides, and the

Golgi, where the carbohydrate chains are elongated. Certain glycosyl residues undergo epimerization and sulfation. As the protein passes through the ER, it is folded into the mature protein. The protein components of the molecule are regulated by all of the known modalities, including transcription rate, alternative promoters, splicing differences, half life of the mRNA and degradation of improperly folded protein. The addition of post-translational modifications involves hundreds of specific enzymes that modify the protein core by *O*- or *N*-glycosylation, tyrosination, hydroxylation, or sumoylation with additional modification, elongations, epimerizations and sulfations of the glycosyl residues. The secretory machinery of a connective tissue cell is specialized for a high rate of synthesis of specific ECM molecules, with the entire cell being ready to provide the necessary amino acids (large amounts of glycine and proline for collagen), with the support of the required enzymes for modifications of the amino acid and glycosyl residues. In addition, the structures of the secretory apparatus, the ER, Golgi apparatus and secretory granules must all be in place. In chondrocytes, there is a degree of compartmentalization of matrix synthesis in the cell, with aggrecan and collagen being synthesized in separate domains.[19]

Fibrillar collagens have propeptides at each end of the molecule. At some time after the fibrillar collagen types I, II, III and V leave the Golgi apparatus, the propeptides are removed by specific proteases, the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases. The propeptides act to help align the three polypeptide chains for trimer formation and are thought to help keep the protein soluble during synthesis and may even play a role in fibrillogenesis. For type IIB collagen, the NH_2-propeptide also functions separately to inhibit the invasion of endothelial cells and osteoclasts into cartilage[20,21] and the COOH-propeptide is found as a part of the ECM in the growth plate.[22] As there is so much collagen synthesis during organogenesis and repair, collagen propeptides are found in the serum and urine and can be used as biomarkers of synthesis.[23–25]

After formation of the collagen fibrils, intermolecular cross-links are formed between hydroxylated lysine residues, forming an aldehyde. Complex cross-links are formed in collagen (pyridinolines derived from three lysine residues) and elastin (desmosines derived from four lysine residues). The cross-links confer physical and mechanical properties to the fibrils and provide the tensile strength fundamental to the structural role of collagen fibrils in connective tissues.[26] The enzyme lysyl oxidase is required for cross-link formation; it is copper dependent and is most active against native collagen fibrils.



Shoulders MD, Raines RT. 2009.
Annu. Rev. Biochem. 78:929–58

Figure 1.4 Collagen biosynthesis. Biosynthetic route from the cell to collagen fibers, for example here, the major component of skin. Size and complexity are increased by post-translational modifications: hydroxylation of lysine and proline, rearrangement of disulfide bonds, and self-assembly. Oxidation of lysine side chains leads to the spontaneous formation of hydroxylysyl pyridinoline and lysyl pyridinoline cross-links between collagen molecules in the matrix.[4]

Deficiency in copper or in lysyl cross-links results in lathyrism, characterized by poor bone formation and strength, hyperextensible skin, weak ligaments and increased occurrence of aortic aneurysms. Lysyl oxidase is also associated with cancer, being responsive to hypoxia-inducible factors, and may be involved in initiation or perpetuation of metastases.[27] Figure 1.4 is a schematic of collagen synthesis in skin.

1.3 ADDITIONAL MATRIX MOLECULES IN SPECIALIZED TISSUES

1.3.1 Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans are a diverse family of GAG-bearing protein cores that include the syndecans, the glypicans, perlecan, agrin and collagen XVIII. They play key roles during normal processes of development, tissue morphogenesis and wound healing. As key components of basement membranes in organs and tissues, they also participate in selective filtration of biological fluids, in establishing cellular barriers and in modulation of angiogenesis. Heparan sulfate is a unique polymer of *N*-acetylgucosamine and glucuronic acid, modified by specific enzymes to generate biologically active structures. The negatively charged heparan sulfate chains bind to growth factors.

1.3.1.1 Perlecan. Perlecan is also called heparan sulfate proteoglycan 2 and is present in basement membranes, mesenchymal cells and vascular cells. However, it is also present in the musculoskeletal tissues, cartilage, meniscus and intervertebral disc, which are devoid of basement membranes and are avascular. The mature core protein is modular, containing 1 sperm protein-enterokinase-agrin (SEA) module, 4 EGF-like domains, 22 Ig-like C2 domains, 3 laminin G-like domains, 3 laminin IV type A domains, and 4 low-density lipoprotein receptor class A domains. Functions of perlecan include modulation of angiogenesis,[28] solute filtration,[29] growth factor delivery *via* heparan sulfate chains,[17] initiation of chondrogenesis,[30] regulation of cell adhesion and fibrillogenesis.[31] A knockout of perlecan in mice primarily results in developmental abnormalities of the heart, brain and cartilage.

1.3.1.2 Syndecans. Syndecan family members classically link the functions of the cell surface heparin-binding growth factor receptors to the cytoskeleton, facilitated by their uniqueness as transmembrane heparin sulfate proteoglycans (HSPGs). They act as high capacity, low affinity co-receptors to their cognate low capacity, high affinity receptors.[32] They can be processed to release the ECM domain of the syndecan into the matrix.

1.3.2 Matricellular Proteins

Another prominent group of ECM proteins are the matricellular proteins. These are ECM proteins that modulate cell–matrix interactions and cell functions but do not seem to have a direct structural role. The family includes thrombospondin-1, thrombospondin-2, osteopontin/Spp1, osteonectin/Sparc, periostin, tenascin C and tenascin X. Expression of matricellular proteins is usually high during embryogenesis, but nearly absent during normal postnatal life. Interestingly, they reappear in response to injury.[33] The cartilage oligomeric protein (COMP) is a member of the thrombospondin gene family (Tsp5). Thrombospondin-2 is involved in collagen fibrillogenesis.[34]

Osteopontin (also called secreted phosphoprotein 1 and bone sialoprotin 1) is a good example of this group of proteins. It is expressed in many tissues and cell types and found in body fluids.[35] The secreted protein is heavily modified post-translationally by *O*-glycosylation, sulfation, and serine/threonine phosphorylation.[36] The functional domains include an integrin attachment motif (GRDGS), thrombin cleavage site, cryptic integrin attachment motif and a mineral binding polyaspartate region. While osteopontin binds tightly to hydroxyapatite and forms an integral part of mineralized bone matrix, it also acts as a cytokine involved in enhancing production of INF-gamma and IL-12 and reducing IL-10 and is essential in the pathway that leads to type I immunity.[37] It is also known to play roles in tissue repair, regulation of bone metabolism, inflammation and immunity.[38]

1.3.3 Elastic Tissues

Many tissues are elastic in nature and, while having many of the same ECM molecules as other tissues, the protein elastin and its associated proteins provide the structural and functional components that allow repetitive distending/relaxing or passive lengthening/shortening movements endowing the tissues with flexibility and rubber-like extensibility. Elastic fibers are solid, branching and unbranching, fine and thick, rod-like fibers (as in nuchal and other elastic ligaments), or they occur as concentric sheets or lamellae (in blood vessels), or arranged in three dimensional meshworks of fine fibrils, as in elastic cartilage of the ear and larynx, or they may occur as combinations of these, as seen in the skin and the lungs. The predominant molecule in elastic fibers is elastin, but other proteins are also involved, such as fibrillins[39] and a smaller glycoprotein, microfibril-associated glycoprotein (MAGP).[40] A single gene encodes elastin, albeit with alternatively spliced products; fibrillin is encoded by two genes (fibrillin-1

and fibrillin-2); MAGP is a single gene. Elastic fibers are extremely inert and difficult to extract from the ECM. While elastic fibers are fairly resistant to degradation, MMP-12 is the elastase isolated from macrophages.[41] The fibers (particularly the fibrillins) are now known to bind to TGFβ family members[42] and play an active role in tissue morphogenesis and response to injury.

1.3.4 Calcified Tissues

Calcification (or biomineralization) is the process by which hydroxyapatite (calcium phosphate, $CaPO_4$) is deposited in the ECM, primarily on the fibrillar collagen. Physiological mineralization occurs in hard tissues, particularly the bone, the hypertrophic zone of the growth plate, and dentin, whereas pathological mineralization occurs in soft tissues such as tendon, kidney, blood vessels and synovial joints. Therefore, the deposition of mineral in the ECM of tissues must be tightly regulated.[43] In addition, the amount of mineralization must be critically regulated, as low bone mineral density leads to osteoporosis, the most prominent bone disease. The first step in mineralization is the formation of hydroxyapatite matrix vesicles[44] (or potentially related exosomes)[45] that bud from the surface membrane of hypertrophic chondrocytes, osteoblasts and odontoblasts. This is followed by propagation of hydroxyapatite into the ECM and its deposition between collagen fibrils. Extracellular inorganic pyrophosphate, provided by extracellular nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) and progressive ankylosis gene product (ANKH) inhibits hydroxyapatite formation. Tissue non-specific alkaline phosphatase (TNAP) hydrolyzes the pyrophosphate and provides inorganic phosphate to promote mineralization. Cellular secreted proteins direct the initiation and directional growth of the mineral phase.[46] In addition, as the calcium and phosphate in body fluids are always in a metastable equilibrium, cells and tissue that are not intended to mineralize must be protected from doing so by expression of specific inhibitory proteins.

Osteoblasts drive intramembranous ossification and ossification during turnover of bone. Osteoblasts express a number of genes that code for bone matrix components and enzymes involved in bone synthesis, including type I collagen, tissue non-specific alkaline phosphatase (TNAP), bone sialoprotein, osteocalcin and osteopontin: many of these proteins are under the control of the transcription factor Runx2.[46] Bone sialoprotein, among others, can nucleate the precipitation of hydroxyapatite through a specific binding to collagen and to hydroxyapatite.[47] Osteoblasts produce bone-specific matrix onto

existing ECM and, as the bone matrix continues to grow, the osteoblasts get entrapped in the matrix and terminally differentiate into osteocytes or undergo apoptosis.

Elongation of the bone requires the cartilage growth plate to allow growth by the process of endochondral bone formation. Initially, a cartilaginous template is formed made up largely of type II fibrillar collagen and the proteoglycan aggrecan. Through a very controlled process, the chondrocytes initially proliferate, then cease proliferation and begin to enlarge to become hypertrophic chondrocytes. Hypertrophic chondrocytes express a very specific complex pattern of genes that prepare the cartilage matrix to be vascularized and mineralized by means of matrix remodeling.[48] Simply, hypertrophic chondrocytes express the transcription factor Runx2 and make many of the same proteins as osteoblasts, including TNAP and bone sialoprotein and the hypertrophic-specific collagen, type X. However, in addition, hypertrophic chondrocytes produce the enzymes necessary to remodel the matrix, primarily MMP13 and MMP9. As will be discussed below, MMPs can liberate and activate growth factors from the ECM, including fibroblast growth factors (FGFs)[49] and vascular endothelial growth factor (VEGF), as well as degrade the cartilaginous matrix. Osteoblasts and osteoclasts are recruited to remodel the matrix and hypertrophic chondrocytes bud off matrix vesicles containing the proteins required for mineralization. The hypertrophic chondrocytes ultimately undergo apoptosis;[50] however, studies indicate that some hypertrophic chondrocytes may also transdifferentiate into osteoblasts.[51]

Inhibitors of mineralization are of critical importance. Most cells, unless injured or aged, do not produce the specialized ECM components and enzymes necessary to nucleate hydroxyapatite mineral formation. The serum proteins fetuin and gamma-carboxyglutamic-rich protein (MGP) help to maintain a high metastable concentration of calcium phosphate and inhibit ectopic calcification.[52] Chondrocytes (other than hypertrophic chondrocytes) and vascular smooth muscle cells synthesize high levels of MGP.

1.4 SURPRISES IN THE ECM – LATENT FUNCTIONS OF MATRIX MOLECULES

1.4.1 Degradative Enzymes in the ECM: MMPs and ADAMs

Enzymes resident in the ECM are required for normal turnover and degradation of the matrix. In some instances, the balance between anabolic and catabolic events is lost, and matrix is destroyed, as classically

seen in diseases such as osteoarthritis, where cartilage is degraded. It is also well established that tumor initiation, progression and invasion are a consequence of a complex cross-talk between different cell types within the tumor microenvironment and are characterized by ability of malignant tumors to destroy matrix barriers, permitting invasion into the surrounding connective tissues, intravasation and extravasation, and metastasis to distant organs. The enzymes responsible for matrix turnover and degradation are called matrix metalloproteinses (MMPs or matrixins) and also include the adamalysins ADAMs (a disintegrin and metalloproteinase domain) and ADAMTS (with an additional thrombospondin domain). The MMPs are a specific group of 25 enzymes named for their dependence on metal ions for catalytic activity and their potent ability to degrade structural proteins of the ECM (Table 1.3). A typical MMP has a multi-domain structure that includes a signal peptide domain (for secretion), a propeptide domain (often a latent form), and a catalytic domain. The ADAMs and ADAMTS enzymes are often membrane bound and include the additional domains described above. There are currently 33 members of the ADAM family and 5 members of the ADAMTS family.[53] Figure 1.5 demonstrates some of the enzymes and cleavage sites for ECM molecules used in cartilage turnover and degradation.[2]

MMPs are secreted as latent enzymes and require activation, which is tightly regulated to prevent tissue damage. The activities of most MMPs are very weak or negligible in normal steady-state tissues, but rapidly increase in response to inflammatory and oxidative stimuli. Their activity can be regulated at four levels: induction of MMP genes, vesicle trafficking and secretion, activation of latent proforms, and complexing with specific endogenous tissue inhibitors of metalloproteinases (TIMPs). Most MMPs are activated in the ECM. The balance between production, activation and inhibition of MMPs is critical in maintaining ECM integrity. When proteolytic activity is greater than inhibition caused by TIMPs or other inhibitors, ECM breakdown occurs. Conversely, if inhibitors are too strongly expressed and proteolysis is restricted, there is a build up of ECM proteins, with fibrosis.

1.4.2 Matrixins – Matrikines and Matricryptins

Once the ECM is established, the function and action of the matrix begins. The ECM is highly responsive and communicative with the cells and other parts of the tissue. Bioactive fragments are released from full-length proteins by limited proteolysis catalyzed by a variety of enzymes such as cathepsins,[54] plasminogen activator/plasmin

Table 1.3 Human MMP members and principal biological effects.[a]

Group name	MMP	Substrates	Biological effects
Collagenase			
Collagenase-1	MMP-1	Collagens I, II, III, VII, X, gelatin, proteoglycan, link protein, entactin, tenascin	Keratinocyte migration and reepithelialization, release of bFGF, platelet aggregation, cell proliferation, pro- and anti-inflammatory, osteoclast activation, cell migration, enhanced collagen affinity, apoptosis, increased bioavailability of TGF
Collagenase-2	MMP-8	Collagens I, II, III, gelatin, proteoglycan, link protein	
Collagenase-3	MMP-13	Collagens I, II, III, IV, IX, X, XIV, proteoglycan, fibronectin, tenascin	
Gelatinase			
Gelatinase A	MMP-2	Gelatin, collagens IV, V, VII, XI, laminin, fibronectin, elastin, proteoglycan, link protein	Neurite outgrowth, generation of angiostatin-like fragment, enhanced collagen affinity, epithelial cell migration, tumor cell resistance, generation of vasoconstrictors, increased bioavailability of TGFβ pro-inflammatory, recruitment of osteoclasts
Gelatinase B	MMP-9	Gelatin, collagens III, IV, V, elastin, entactin, link protein	
Stromelysin			
Stromelysin-1	MMP-3	Proteoglycan, collagens III, IV, IX, X, laminin, fibronectin, gelatin, tenascin, link protein, elastin	Mammary epithelial cell apoptosis and alveolar formation, generation of angiostatin-like fragment, release of bFGF, increased bioavailability of IGF1 and cell proliferation, increased bioavailability of TGFβ, increased cell invasion, anti-inflammatory
Stromelysin-2	MMP-10	Collagens III, IV, V, fibronectin, laminin, proteoglycan, link protein, elastin	
Stromelysin-3	MMP-11	Fibronectin, laminin, proteoglycan, gelatin	

Membrane-type			
MT1-MMP	MMP-14	Collagens I, II, III, gelatin, proteoglycan, fibronectin, laminin	Cell migration, kidney tubulogenesis, epithelial cell migration, reduced cell adhesion and spreading, embryo attachment to uterine epithelia
MT2-MMP	MMP-15	Fibronectin, tenascin, entactin, aggrecan, perlecan, laminin	
MT3-MMP	MMP-16	Collagen III, gelatin, fibronectin	
MT4-MMP	MMP-17	?	
MT5-MMP	MMP-24	Proteoglycan	
MP6-MMP	MMP-25	Gelatin	
Others			
Matrilysin	MMP-7	Proteoglycan, gelatin, fibronectin, tenascin, elastin, collagen IV, laminin, link protein	Adipocyte differentiation, generation of angiostatin-like fragment, increased bioavailability of IGF1 and cell proliferation, epithelial cell migration, increased bioavailability of TGFβ, disrupted cell aggregation and increased cell invasion, Fas-receptor mediated apoptosis, pro-inflammatory, osteocleast activation, vasocontrictionand cell growth
Metalloelastase	MMP-12	Elastin	
Collagenase-4	MMP-18	Collagen I	
RAS I-1	MMP-19	Tenascin, gelatin, aggrecan	
Enamelysin	MMP-20	Enamel, gelatin	
XMMP	MMP-21	?	
No name	MMP-23	?	
Matrilysin 2	MMP-26	Collagen IV, fibronectin, gelatin	
No name	MMP-27	?	
Epilysin	MMP-28	Casein	

[a]From Gargiulo *et al.*, 2014.

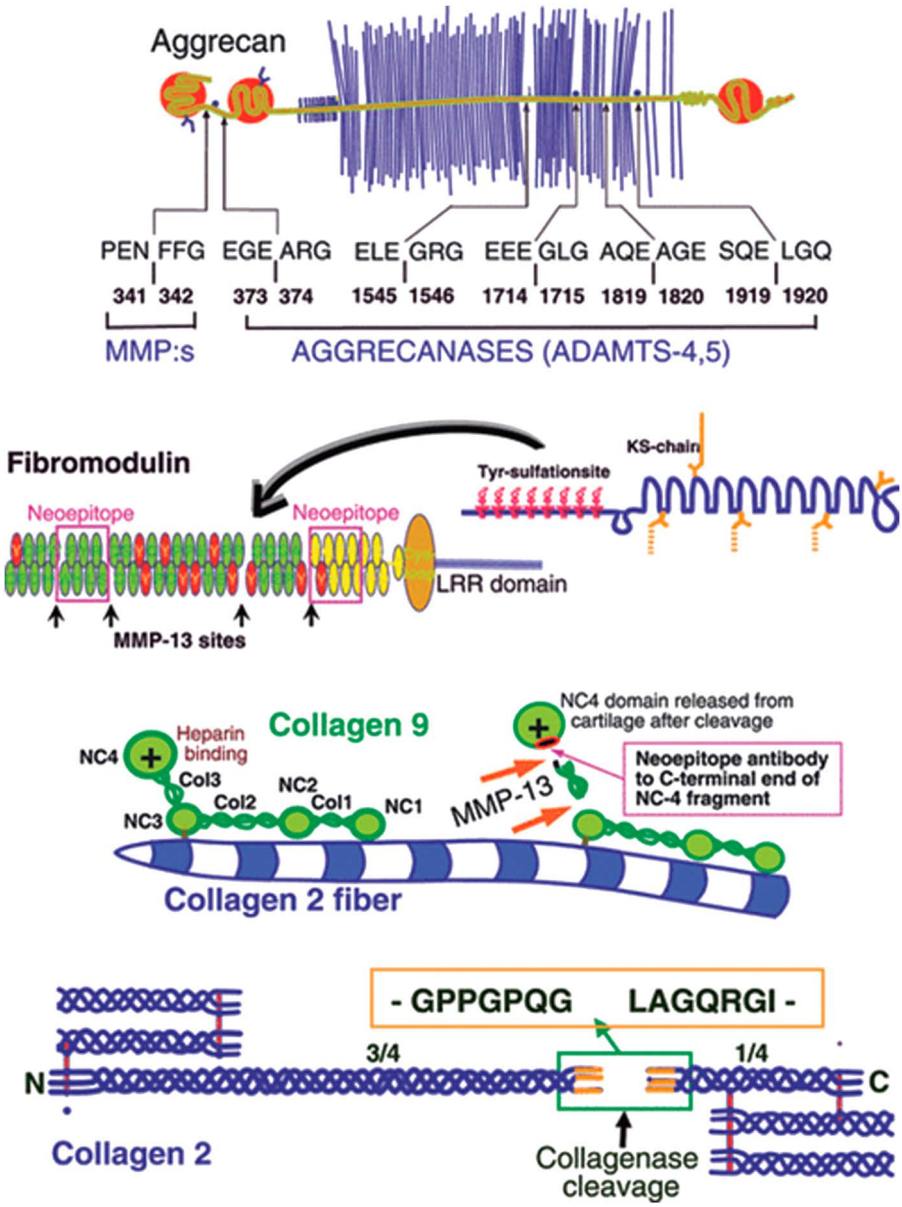


Figure 1.5 Illustrations of some specific events in cartilage breakdown, indicating specific cleavage sites and enzymes that are known to induce this cleavage at the tissue level. The degradation of aggrecan, fibromodulin, type IX and type II collagens are indicated.[2]

system[55] and MMPs. Generally these fragments provide new activities that are different from the intact molecules. MMP-2, MMP-7 and MMP-13 produce endostatin, an anti-angiogenic peptide from type XVIII,[56] MMP-9 produce tumstatin, an anti-angiogenic fragment of type IV collagen,[57] MMP-7, MMP-9, MMP-12 produce elastokines.[58] These new bioactive fragments are called matrikines and matricryptins (or elastokines if from elastin). In addition, heterotypic binding, cell-mediated mechanical forces, denaturation, multimerization, self-assembly and reactive oxygen species may also expose bioactive matricryptic sites in the ECM.[59,60] The release and activity of these new fragments adds an additional level of regulation to the ECM, modulating angiogenesis, cancer, fibrosis, inflammation, neuro-degenerative diseases and wound healing. There is a significant degree of interest in using these biologically active molecules as therapeutic agents.

Matrikines are defined by Maquart *et al.*[61] and Swindle *et al.*[62] as signaling elements that exist as subcomponents of ECM proteins and bind to cell surface receptors that belong to the cytokine, chemokine, ion channel or growth factor receptor family. These ligands (for example, tenascin EGF-like repeats) are encrypted within matrix components and modulate cellular responses mediated by growth factor receptors, and thus are considered as constrained to a surface, restricting their effect to the cells located in their vicinity. Most authors use matrikine and matricryptin interchangeably, with the definition being that they are "proteinase-generated fragments of matrix macromolecules that display cryptic bioactivities not manifested by the native, full length form of the molecule".[63] Peptides released from growth factors, chemokines and cytokines are not considered matrikines and are a separate class of bioactive fragments,[64] Recard-Blum and Salza suggest including fragments of ECM regulators (MMPs, ADAMs, and cross-linking enzymes) and ECM-affiliated proteins (*e.g.* mucins, galectins, semaphorins) as matricryptins. The ectodomains of membrane collagens (XIII, XVII, XXIII, and XXV) that are shed from the cell surface *in vivo* and released in the extracellular milieu, where they behave like cytokines, are martricryptins. The ectodomain of collagen XIII module cell adhesion, migration and proliferation and increased shedding of collagen XVII correlates with a decrease in keratinocyte motility. The ectodomains of syndecans 1-4, which are shed from the cell surface *in vivo*, contain adhesion regulatory domains and are also matricryptins.[65] Metastatin, comprised of proteolytic fragments of the link protein and the aggrecan core protein, inhibits angiogenesis and tumor growth and is a matrikine.[66] Oligosaccharides cleaved from hyaluronan[67,68] also belong to the matricryptin family and promote inflammation and improve wound healing.

The sources of matrikines vary, with each tissue having a specific set of ECM molecules and a specific set of MMPs that are capable of generating bioactive fragments. Collagens and proteoglycans,[69] elastin[58,70] and laminins[71] are major sources of matrikines. Matricellular proteins (*e.g.* SPARC, osteopontin) are additional sources. Some ECM proteins are cleaved into several fragments with either similar (*e.g.* collagen IV) or opposite activities for example SPARC, which generate both anti-angiogenic and pro-angiogenic fragments.[72] Most of the biological activities of ECM fragments are mediated by integrins, heparan sulfate proteoglycans and growth factor receptors.

Matrikines are usually produced under conditions where the producing enzyme has been activated, such as wound healing, cancer, inflammation, *etc.* Therefore the functions range from participation in angiogenesis (either pro- or anti-), ECM synthesis and remodeling and ECM assembly. In the early stages of angiogenesis, the degradation of ECM occurs in response to angiogenic stimuli. As a consequence, matrix molecules are degraded or partially modified, soluble factors are released and cryptic sites are exposed. Migration of cells through the basement membrane is a requirement for metastasis. Type IV collagens and perlecan are major components of the basement membrane: MMP cleavage of type IV collagen yields tumstatin, canstatin and arresten derived from the α3, α2 and α1 chains of the collagen;[73] tetrastatin, pentastatin and hexastatin are derived form the α4, α5 and α6 chains of type IV collagen, and endorepellin is derived from perlecan.[74] Peptides from the α4 and α5 laminin chains exhibit anti-microbial activity against *Escherichia coli* and *Staphylococcus aureus*.[75] ECM fragments also influence obesity and adipogenesis. Endostatin induces weight reduction in a murine model of obesity and regulates adipose tissue mass.[76] Elastin peptides regulate insulin resistance in mice[77] and endotrophin, a cleavage product of type IV collagen secreted by adipocytes, may play a role in obesity-related cancers.[78] Endotrophin also promotes tumor progression and fibrosis,[79] whereas endostatin has antifibrotic activity in dermal and pulmonary fibrosis.[80] Many other examples exist and are tissue specific (see Ricard-Blum and Salza, 2014 for a review and an exhaustive list of ECM bioactive fragments, their source and function).[64]

1.4.3 Growth Factors

Growth factors can be sequestered in the ECM either by binding to protein moieties or by binding to negatively charged carbohydrate chains. We have known for many years that specific cells produce

growth factors at a given time and they are stored in the ECM for later use. Type IIA procollagen was one of the first ECM proteins shown to bind growth factors TGFβ, BMP-2 and BMP-7.[81] Type IIA procollagen retains the NH_2-propeptide in the ECM containing a VWF-C domain that binds to BMPs and TGFβs with high affinity (10^{-9} nM). Chordin, an inhibitor of BMPs in zebrafish[82] and decapentaplegic, which binds to BMPs in *Drosophila*, also bind *via* the same VWF-C domain.[83] These growth factors can be released by cleavage of the collagen or chordin by MMPs, releasing active BMPs.[82,84] Many of the effects of the matrix protein fibrillin are mediated through its binding to TGFβ, sequestering and then releasing the growth factor.[42] The sequestering of growth factors in the ECM allows growth factor gradients to be established and provides for a reserve supply of growth factors for later use.

In the late 1980's experiments by Comper and colleagues[85] found that the charged groups of the glycosaminoglycan chains of proteoglycans in cartilage did not participate in the water flow resistance in cartilage, as had been previously believed. Subsequently, it was discovered that these charged GAG chains bound growth factors.[86] These findings led to analysis of all GAG chains for growth factor binding and it was found that FGFs bound to HSPGs (perlecan and syndecans) at the cell surface and facilitated interaction with the actual FGF receptor.[87] Heparin in the matrix and bloodstream also binds to growth factors and has been used extensively for isolation.[88] The angiogenic growth factor VEGF is stored extracellularly, binding to the cell surface of ECM (fibronectin, HSPGs) and various MMPs.[74] Table 1.4 shows some growth factors that bind to perlecan.

Table 1.4 Interactions of perlecan with growth factors and morphogens.[a]

Growth Factor	Interacting Perlecan Moiety
FGF2	Heparan sulfate chains Domain I
FGF7	Domain III and Domain V/endorepellin
FGF10	Heparan sulfate chains, specific microdomains
FGF18	Domain III
VEGF	Heparan sulfate chains; co-localization with perlecan in tumor angiogenic vessels
PDGF	Heparan sulfate chains and Domains III and IV
Progranulin	Domain V/endorepellin
Hedgehog	Domain V/endorepellin of *Trol in Drosophilia*
TGFβ/Wnt	Protein core/UNC-52 *Trol in Drosophilia*
IL-2	Heparan sulfate chains

[a]From Whitelock *et al.*, 2008.

1.5 CONCLUSIONS

The ECM is a dynamic and response component of almost all tissues, ranging from blood vessels to cartilage. Specific combinations of molecules provide exquisite specialization of the ECM. ECM can initiate and respond to mechanical forces, developmental programs, injury and disease, and if not controlled properly can participate in progression of disease. The challenge to the tissue engineer is clearly to construct a matrix that has all of the necessary functional properties of the native matrix, and potentially to confer the complex properties necessary for the matrix to respond to the environment, whether it is normal homeostasis or a drastic insult. Many of the biologic aspects of matrix are already being used in the construction of tissue-engineered matrices, such as engineering the release of bioactive peptides. These aspects of ECM will be explained in detail in the following chapters.

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