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Jaroslava Halper *Editor*

Progress in Heritable Soft Connective Tissue Diseases

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Editor

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Contents

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Introduction

1

Jaroslava Halper

Marfan and Ehlers-Danlos syndromes are the two best known and most studied heritable disorders of soft tissues. The contrast between what was known about clinical presentation and symptomatology, pathology, and mode of inheritance of what we call Marfan syndrome today when it was described first in a case report in 1896 by Antoine Marfan himself [1] and later as dystrophia mesodermalis typus Marfan in 1931 [2, 3], and what we know today about its biochemistry, genetics, and clinical picture is truly astounding. Today we have quite extensive (and unprecedented) knowledge of various biochemical and genetic aspects of the numerous types of Ehlers-Danlos syndrome, and we need to give credit to Job Janszoon Van Meek'ren, a Dutch surgeon, who gave a very apt, first documented description of a person suffering from Ehlers-Danlos already in 1657 [4, 5]. Because of rapidly expanding knowledge about heritable soft tissue disorders which needs to be constantly updated, we conceived of a volume which, rather than being a comprehensive tome of information that would rapidly become obsolete, would bridge basic science and clinical application, and so would serve as a reference handbook for basic scientists and clinicians seeing patients, and as a stepping stone for new investigations and studies. Rather than simply rehashing facts about the composition and biochemistry of the connective tissue and extracellular matrix, we tried to connect individual components to specific aspects of various soft tissue disorders and to the actual or potential treatment of them in the first four chapters (Chaps. 2–5).

With the goals of finding some unifying features connecting these disorders and of offering an overview of the state of the art, and the newest ideas on treatment and factors involved in pathogenesis, we brought together a group of prominent scientists and clinicians working on various aspects of heritable disease of soft tissues and/or problems of connective tissues. One of the most fascinating aspects of these diseases is the connection between the embryonic development of cardiac valves (and other components of the cardiovascular system) and that of tendons. It is the fibrosa layer of cardiac valves which is situated away from the blood flow and which resembles tendons in several aspects: both structures express tenascin, express types I, III, and V collagens during remodeling, and are composed of parallel type I collagen fibers after they achieve maturity [6]. It then follows that their fates would be intertwined, and indeed they are.

TGFβ is the factor, at least at this juncture, whose involvement in development and physiology of soft tissues explains, at least in part, that many of these disorders are interconnected though the primary pathophysiological events, such as gene mutations, may be different in each disorder.

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While Cook and Ramirez describe different aspects of Marfan syndrome in Chap. 6, including the (indirect) role TGFβ plays in its pathogenesis, Jones et al. (Chap. 8) look at the problem from the other side of the fence – why would TGFβ be involved not only in Marfan syndrome but also in several other disorders of the cardiovascular system, e.g., in Loeys-Dietz syndrome described in Chap. 7 by none other than Loeys, Dietz and one of their colleagues.

In two related and complementary chapters on Ehlers-Danlos Malfait and dePaepe (Chap. 9), and Miyake et al. (Chap. 10) discuss the newest developments in discovering new and uncommon forms of Ehlers-Danlos. From their description it is clear that the clinical and biochemical differences among the different types of Ehlers-Danlos may represent a diagnostic challenge for clinicians and require an acute diagnostic acumen even today, in the time of advanced imaging technology and clinicopathological/biochemical analysis!!

Chapter 11 brings forward the recognition that there are many variations on the theme of cutis laxa either as a primary heritable disorder or as a component of a metabolic disease. While there are many overlaps among those forms of cutis laxa, the authors describe helpful diagnostic signs and approach, and emphasize the essential role of astute clinical observation and examination of the patient, just like in the time when individual diseases were recognized, distinguished from each other and classified in "the pretechnology age" on the basis of thorough clinical examination. Chapter 12 by Bushby et al. reviews certain myopathies which are the consequence of mutations in the genes for collagen type VI. These myopathies have been recognized as separate disorders only relatively recently, and because of the type of the underlying mutations, joint laxity and contractures are common in affected patients as is an increased expression of, yes, you guessed correctly – of TGFβ.

These findings emphasize the crucial role of TGFβ in the pathogenesis of many diseases affecting soft tissues, also suggesting that regulation of the level and/or bioactivity of TGFβ may in effect be utilized in the management of these diseases as well. And this, of course, has been shown to be of some use in Marfan and some other syndromes as well, beside surgical intervention and physical therapy.

New biomedical methodology led to the creation of knockout mice which, in turn, enabled the development of several models of heritable disorders of soft tissues. The most relevant mouse models of disorders in expression of molecules participating in the assembly, growth, and maturation of collagen fibrils, such as fibril-forming collagens themselves, regulatory factors like Fibril Associated Collagens with Interrupted Triple helices (FACITs) and small leucine rich proteoglycans (SLRPs) are reviewed extensively in Chap. 13 together with consequences of deficiency in various growth factors and transcription factors, and proteoglycans other than SPLRPs. The short gestation time, large offspring, small size and relative ease of genetic manipulation make mice good and, comparatively speaking, inexpensive material for genetic, biochemical, and morphological studies of heritable soft tissue disorders. Another big advantage is the great degree of external control of expression which provides a high degree of reproducibility in large numbers of animals from one experiment to another.

However, what not many scientists realize is that domestic animals suffer from similar diseases as well as humans. Marfan syndrome was described in cattle, and a mild form of Ehlers-Danlos in horses, dogs and cats (Chap. 14). Though the use of domestic animals as models of human diseases is not common in science because of certain hurdles, such as dependence on random, spontaneous (and not quite common and reproducible) occurrence of such disease, the large size and long life of some of these animals, their relatively low offspring number, and expensive upkeep, it is becoming obvious that some species can be utilized to great benefit. In particular, dogs are perhaps more suitable for studies of at least human disease as they develop many diseases analogous to human diseases, among them a form of Ehlers-Danlos syndrome, and share the same lifestyle, through no fault of their own [7].

I hope that this volume will be of use to clinicians, geneticists, biochemists and other basic

scientists, and, of course, to medical students and residents in their quest for better understanding of pathogenesis of heritable disorders of soft tissues and for better types of treatment of these somewhat elusive entities.

I would like to thank all contributors for their hard work and patience with the slow process of putting this volume together. Many thanks go to Thijs van Vlijmen, the publishing editor at Springer for his guidance, and instant availability when needed.

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Structure, Physiology, and Biochemistry of Collagens

2

Michael J. Mienaltowski and David E. Birk

Abstract

Tendons and ligaments are connective tissues that guide motion, share loads, and transmit forces in a manner that is unique to each as well as the anatomical site and biomechanical stresses to which they are subjected. Collagens are the major molecular components of both tendons and ligaments. The hierarchical structure of tendon and its functional properties are determined by the collagens present, as well as their supramolecular organization. There are 28 different types of collagen that assemble into a variety of supramolecular structures. The assembly of specific supramolecular structures is dependent on the interaction with other matrix molecules as well as the cellular elements. Multiple suprastructural assemblies are integrated to form the functional tendon/ligament. This chapter begins with a discussion of collagen molecules. This is followed by a definition of the supramolecular structures assembled by different collagen types. The general principles involved in the assembly of collagen-containing suprastructures are presented focusing on the regulation of tendon collagen fibrillogenesis. Finally, site-specific differences are discussed. While generalizations can be made, differences exist between different tendons as well as between tendons and ligaments. Compositional differences will impact structure that in turn will determine functional differences. Elucidation of the unique physiology and pathophysiology of different tendons and ligaments will require an appreciation of the role compositional differences have on collagen suprastructural assembly, tissue organization, and function.

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Keywords
Hierarchical structure of tendon • Supramolecular structures of collagens • Collagens I-XXVIII • Fibril-forming collagens • Procollagens • Triple helix • Crosslinking • Fibril-associated collagens with interrupted triple helices (FACIT) • Beaded filament-forming collagen • Network-forming collagens

2.1 Introduction

The composition and structure of tendons and ligaments play important roles in their functions. Tendons attach muscles to bones, and ligaments act to connect bone to bone across a joint space. Both tendons and ligaments are fibrous connective tissues that are composed of cells within an extracellular matrix rich in collagens, proteoglycans, and water. However, the distinct function of each connective tissue is intricately linked to its specific composition and resulting structure. This also can be described for tendon type or location (e.g., axial versus limb, flexor versus extensor) and for regions of tendons (e.g., midsubstance versus enthesis). The composition of tendons and ligaments allows these connective tissues to help guide motion, to resist abnormal displacement of bones and center the actions of several muscles, and to share load and distribute force. The general compositions of tendons and ligaments are similar. Both are composed of water (50–60 % for tendons and 60–70 % for ligaments), collagens (70–80 % dry weight for tendons and more than 80 % for ligaments), and proteoglycans, including small leucine-rich proteoglycans [1]. The collagens present and the supramolecular structures assembled play significant roles in the function of these musculoskeletal connective tissues. Tendons are hierarchical structures with structure and function being closely linked (Fig. 2.1). Tendons are composed primarily of collagen fibrils. Bundles of fibrils are organized as fibers. Fibers are grouped together with tenocytes as fascicles, which are surrounded by a cellular, loose connective tissue, the endotenon. The outer edge of a bundle of fascicles comprising the tendon is demarcated by a contiguous epitenon cover. Structure and composition of such collagen-rich tissues allow for tendons and ligaments to be relatively compliant at low energy, low loading forces, yet to increase in stiffness with increasing forces and loads.

This chapter provides an overview of collagens with a focus on tendons and ligaments. The first section provides an overview of collagens focusing on those collagens found within tendons and ligaments. This is followed by a discussion of collagen synthesis, fibril assembly, as well as fibril growth, and maturation from the assembly of protofibrils to the organization of fibrils into mature fibers. Finally, the tissue structure and function of the collagen-rich extracellular matrix of connective tissues like tendons and ligaments are discussed in the context of differing roles for the collagens in musculoskeletal physiology.

2.2 Collagens

Collagens are proteins that are major components of the extracellular matrix of connective tissues. Members of the collagen family are trimers. They have at least one collagenous or COL domain as well as non-collagenous or NC domains. The number and structure of COL and NC domains are dependent upon the specific collagen type. The importance of these domains will be discussed. Among the genomes of vertebrates and higher invertebrates, there are 28 distinct collagen glycoproteins that are encoded by at least 45 genes. These collagens have been given Roman numeral designations (I–XXVIII) in chronological order of discovery, and they are classified by type based on domain structure and their supramolecular organization. For each collagen type,

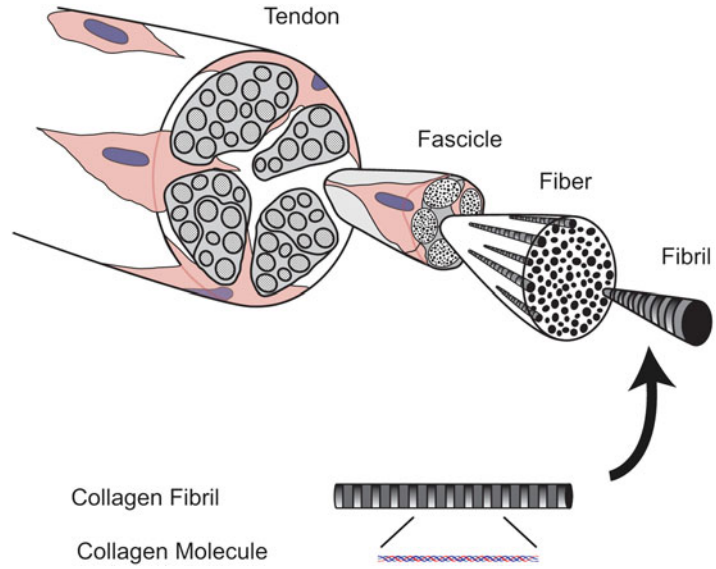

Fig. 2.1 Hierarchical structure of tendon. Tendons are hierarchical structures composed primarily of collagen fibrils. Triple helical collagen molecules assemble to form fibrils. Fibrils bundle together to form fibers. Within the mature tendon, fibers are grouped together with tenocytes within fascicles that are surrounded by a cellular, loose connective tissue, the endotenon. The outer edge of a bundle of fascicles is the epitenon; it contiguously covers the outside of the tendon

each genetically distinct alpha chain is designated by Arabic numerals. The alpha chains of one collagen type are unique from the alpha chains of another collagen type. Each distinct alpha chain is encoded by a different gene, and each distinct alpha chain has its own primary (domain) structure which contributes to the classification by collagen type. For example, the human α1(I) chain is encoded by the *COL1A1* gene and the mouse α1(II) chain by the *Col2a1* gene. Collagens can be homotrimeric; that is, they are composed of three identical alpha chains, like $[\alpha1(II)]_3$ for collagen II. However, collagens also can be heterotrimeric, comprised of alpha chains encoded by different genes of the same collagen type, like $[\alpha1(I)]$ $[\alpha2(I)]_2$ for collagen I. Moreover, it is possible for a single collagen type to have multiple chain compositions, like $[\alpha1(V)]$ $[\alpha2(V)]_2$, $[\alpha1(V)]$ $[\alpha2(V)]$ $[\alpha3(V)]$, or $[\alpha1(V)]_3$ for collagen V.

Collagens may be grouped into classes based upon their suprastructural organization (Table 2.1). Some collagens are fibril-forming; for example, collagens I, II, and III. These collagens form fibrils with a distinct 67 nm periodicity. There are also Fibril-Associated Collagens with Interrupted Triple helices (FACIT) collagens that associate with collagen fibrils and interact with collagenous and non-collagenous proteins. Collagens in each category have their own specialized function and contribute to higher order tissue structures. As collagens from various categories assemble together with varying abundance and with co-polymerization with other non-collagenous macromolecules, they contribute to a tissue suprastructure and thus to its function. Likewise, with development and growth as well as repair and remodeling, the relative abundance and localization of different collagenous and non-collagenous macromolecules are major determinants of the structure and function of that tissue. This is certainly true of tendons and ligaments.

In tendons and ligaments, greater than 90 % of the connective tissue is composed of collagen I [1]. While collagen I is the predominant collagen in these tissues, other collagen types and non-collagenous macromolecules add diversity to the matrix and regulate just how fibril and fiber assembly occurs. This ultimately leads to tissue-specific structure and organization. As is the case with tendons by type, location, and even by zone within each tendon, collagen-containing suprastructures function differently based upon their complex macromolecular compositions that include other collagen types as well as

Table 2.1 General classification of collagen types

Classification	Collagen types	Supramolecular structure
Fibril-forming collagen	I, II, III	Striated fibrils
	V, XI	Striated fibrils, retain N-terminal regulatory domains
	XXIV, XXVII	Unknown
FACIT[a] collagens	IX, XII, XIV	Associated with fibrils, other interactions
FACIT-like collagens	XVI, XIX, XXI, XXII	Interfacial regions, basement membrane zones
Network-forming collagens		
Basement membrane	IV	Chicken wire network with lateral association
Beaded filament-forming	VI	Beaded filaments, networks
Anchoring fibrils	VII	Laterally associated anti-parallel dimers
Hexagonal networks	VIII, X	Hexagonal lattices
Transmembrane collagens	XIII, XVII, XXIII, XXV	Transmembrane and shed soluble ecto-domains
	Gliomedins, ectodysplasin	
Multiplexin collagens (Endostatin–XV and -XVIII)	XV, XVIII	Basement membranes, cleaved C-terminal domains influence angiogenesis
Other molecules with collagenous domains	XXVI, XXVIII	Collagenous domains in primarily non-collagenous molecules
	Acetylcholinesterase, adiponectin, C1q, collectins, surfactant protein, others	

[a]Fibril-associated collagen with interrupted triple helix

non-collagenous components. These additional macromolecules may be substantial or only occur in minute quantities. However, typically the architecture and function of tendons and ligaments are determined by the composite structure of collagen suprastructures. In the following section, the collagens contributing to the suprastructural organization of tendons and ligaments will be reviewed.

2.3 Fibril-Forming Collagens

The fibril-forming collagen subfamily includes collagens I, II, III, V, XI, XXIV and XXVII. Collagens I, II, III, V, and XI have been found in tendons and in ligaments [1–3]. These collagens have a long uninterrupted triple helical domain (ca. 300 nm). Fibril-forming collagen genes cluster into three distinct subclasses[4] and this carries over into functional subclasses. Collagens I, II and III are the most abundant proteins in the vertebrate body and are the bulk components of all collagen fibrils. Within tendons and ligaments, type III collagen is found in greater abundance during embryonic development; however, with maturation levels of collagen III decrease, though in the chicken it still persists in the endotenon and tendon sheath [5]. Moreover, in mature tendons and ligaments of rabbits, it has been noted that collagen III comprises 5 % and 10 %, respectively, of all collagen content [6]. Otherwise, collagen I is the predominant collagen of the tendon and ligament mid-substance. It should be noted that collagen II is generally found within the fibrocartilaginous zone of the enthesis site for both tendons and ligament [7, 8]. Collagens V and XI are quantitatively minor collagens found co-assembled with types I, II and III; they are found on the surfaceome (i.e., plasma membrane and pericellular matrix) of tendon fibroblasts [9]. This fibril-forming subclass retains portions of the N-terminal propeptide and is involved in the regulation of fibril assembly.[2] Collagens XXIV and XXVII make up the third subclass and have differences relative to the other fibril-forming collagen types including, shorter helical regions that are interrupted. Their structural organization

and specific roles remain to be elucidated, and they have yet to be found within tendons and ligaments.

The fibril-forming collagens are synthesized and secreted as procollagens. Procollagens contain a non-collagenous C-terminal propeptide and an N-terminal propeptide. The N-propeptide is composed of several non-collagenous domains and a short collagenous domain. The presence of the propeptides prevents premature assembly of collagen molecules into fibrils. The initial assembly of collagen into fibrils is regulated by the processing of the propeptides which involves several enzymes. The C-propeptides are processed by bone morphogenetic protein 1(BMP-1)/tolloid proteinases or furin [10, 11]. The processing of the N-propeptides involves certain members of the a-disintegrin-and-metalloproteinase-with-thrombospondin-like-motifs family (ADAMTS 2, 3 and 14) as well as BMP-1 [12]. Propeptide processing enzymes have specificity for each different collagen type.[10, 12] Propeptide processing may be complete, thus leaving a collagen molecule with one large central triple helical domain and terminal, short non-collagenous sequences termed the telopeptides, as is the case for collagens I and II. However, with collagens III, V, and XI, processing can be incomplete, with the retention of a C-telopeptide and a partially processed N-propeptide domain, which have been implicated in the regulation of fibrillogenesis [13–17]. After propeptide processing, collagen molecules self-assemble to form striated fibrils with a periodicity of 67 nm. Within each fibril, collagen molecules arrange longitudinally in staggered arrays. Thus, a gap occurs between the ends of neighboring molecules, and this gap-overlap structure is present in all collagen fibrils with a 67 nm D-periodic banding pattern. This is presented schematically in Fig. 2.2a.

Collagen fibrils are heterotypic. That is, they are assembled from mixtures of two or more fibril-forming collagen types. In tendons and ligaments, collagens I and III are the quantitatively major fibril-forming collagens with collagen II present within fibrocartilaginous regions. Heterotypic collagen fibrils of tendons and ligaments also contain quantitatively minor amounts of collagens V and XI; as mentioned above, these regulatory fibril-forming collagens are characterized by partial processing of N-propeptide domains. The N-propeptides have a flexible or hinge domain (NC2) between the triple helical domain (COL1) and a short triple helical domain (COL2). The N-terminal domain (NC3) is composed of a variable domain and a proline/arginine-rich protein (PARP) domain. Partial processing removes the PARP yet retains the hinge, COL2, and variable domains [18–20]. The regulatory fibril-forming collagens co-assemble with the major fibril-forming collagens in the heterotypic fibril; however, the N-terminal domain of the regulatory fibril forming collagens cannot be integrated into the staggered packing of the helical domains. The rigid COL2 domain of the collagen V or XII molecule can project toward the fibril surface in the gap region of the assembled fibril (Fig. 2.2b). Recent findings have demonstrated that alteration in collagens V and/or XI affects tendon fibril assembly during tendon development. Changes include altered fibril structure, decreased fibril number and abnormal fibril and fiber organization [21]. Thus, interactions between the fibrillar collagens affect the organization of collagen fibrils within collagen-rich tissues like tendons and ligaments [22].

2.4 Fibril-Associated Collagens with Interrupted Triple Helices (FACIT)

FACIT collagens closely interact with fibril-forming collagens. These molecules affect the surface properties of fibrils as well as fibril packing. Collagens IX, XII, XIV and XX are FACIT collagens. Type IX collagen is primarily found interacting with collagen II. Collagen IX is also a proteoglycan with covalently attached glycosaminoglycan side chains in cartilage [23]. This is also true with Type XII collagen [24]. Collagens XII and XIV have been found throughout musculoskeletal connective tissues, including tendons and ligaments at various times during development; [25, 26] collagen XIV has been found specifically at the bone-ligament interface in bovine entheses [27] In general, FACIT collagens have short COL

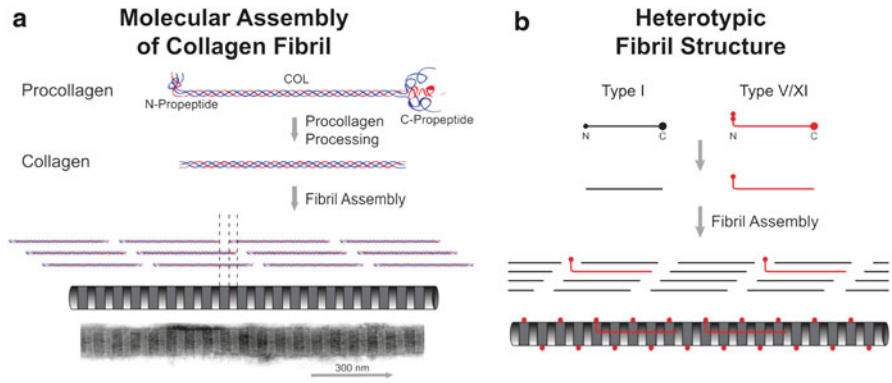

Fig. 2.2 Fibril-forming collagens. (**a**) Fibril-forming collagens are synthesized as procollagens. Procollagens contain a central COL domain and flanking propeptide N-and C-terminal NC domains. Propeptides are processed and the resulting collagen molecules assemble to form striated fibrils. Each fibrillar collagen molecule is approximately 300 nm (4.4D) in length and 1.5 nm in diameter. Within the fibril, the collagen molecules are staggered N to C in a pattern that gives rise to the D-periodic repeat. At the bottom of the panel, a D-periodic collagen fibril from tendon is presented. The characteristic alternating light and dark pattern represents the respective overlap and gap regions of the fibril. (**b**) Collagen fibrils are heterotypic. That is, they are co-assembled from quantitatively major fibril-forming collagens (e.g., I, II, or III) and regulatory fibril-forming collagens (V or XI). Regulatory fibril-forming collagens have a partially processed N-terminal propeptide, retaining a non-collagenous domain that must be in/on the gap region/fibril surface. The heterotypic interaction is involved in nucleation of fibril assembly (This figure has been adapted from Birk and Bruckner [131])

domains interrupted by NC domains with an N-terminal NC domain that projects into the interfibrillar space (Fig. 2.3). FACIT collagens have two C-terminal domains NC1 and COL1 that are believed to interact with the collagen I fibrils. At the N-termini of FACIT collagens, the large globular NC domains protrude from the fibril surface [2]. FACIT collagens along the surface of fibrils have been shown affect fiber suprastructures and tendon biomechanics [25, 26].

The FACIT-like collagens have features in common with FACIT collagen, though they are structurally and functionally unique. This FACIT-like group includes collagens XVI, XIX, XXI and XXII, yet their roles in musculoskeletal connective tissues have yet to be elucidated [28–32].

2.5 Basement Membrane Collagen

Collagen IV is considered a basement membrane collagen. It is the collagenous component of an integrated network of several matrix molecules that form an extracellular matrix that defines the interface between tissues [33, 34], including to some extent musculoskeletal tissues, particularly those that are adjacent to or fed by vasculature. For example, vasculature is found along the sheath, paratenon, and epitenon of tendons in a tendon-specific manner and basically along the "epiligament," or surrounding surface layer of tissue for ligaments [1]. Throughout the body, there are diverse networks of basement membranes composed of many macromolecules that are anatomical site-dependent. Likewise, there are several subtypes of collagen IV which are composed of different stoichiometries of 6 collagen IV-encoding genes COL4A1 through COL4A6 reviewed by Khoshnoodi et al. [35].

2.6 Beaded Filament-Forming Collagen

Collagen VI is ubiquitous within connective tissue; it is found as an extensive filamentous network with collagen fibrils, and is often enriched in pericellular regions. Collagen VI can be assembled into several different tissue forms,

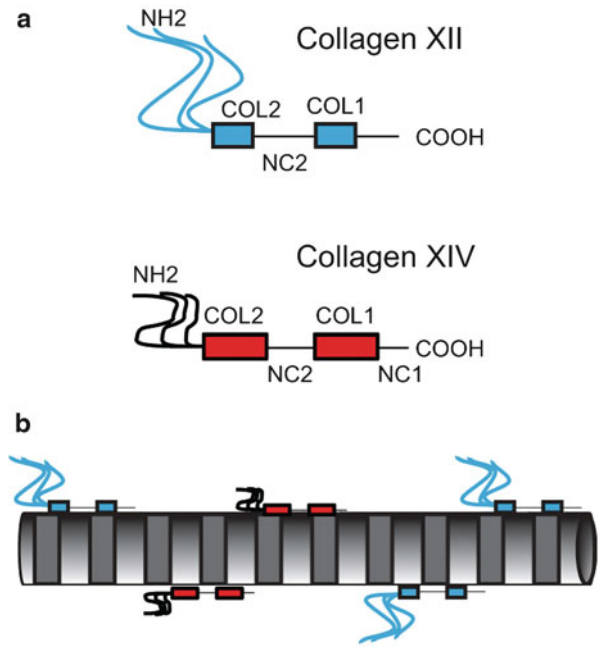

Fig. 2.3 FACIT collagens associate with fibrils. (**a**) The domain structures of FACIT collagens found in tendons and ligaments are illustrated. Note that all FACITs have alternative spliced variants, and collagen XII can have glycosaminoglycan chains attached covalently. The FACIT collagens have 2-3 COL domains and 3-4 NC domains with a large N-terminal NC domain that projects into the inter-fibrillar space. (**b**) The FACIT collagens all associate with the surface of collagen fibrils, including N-truncated isoforms due to alternative splicing in collagen XII. Collagen XII is capable of other non-fibril interactions (not shown) (This figure has been adapted from Birk and Bruckner [131])

including beaded microfibrils, broad banded structures and hexagonal networks [36–38]. Collagen VI interacts with many extracellular molecules including: collagens I, II, IV, XIV; microfibril-associated glycoprotein (MAGP-1); perlecan; decorin and biglycan; hyaluronan, heparin and fibronectin, as well as integrins and the cell-surface proteoglycan NG2. Based on the tissue-localization and large number of potential interactions, collagen VI has been proposed to integrate different components of the extracellular matrix, including cells [39]. Collagen VI also may influence cell proliferation, apoptosis, migration, and differentiation. Thus, collagen VI is involved in the development of tissue-specific extracellular matrices, repair processes and in the maintenance of tissue homeostasis. In musculoskeletal tissue, collagen VI has proven to be essential; mutations have been shown to cause various forms of muscular dystrophy as well as proximal joint contractures involving tendons in humans[40–43] (**See also Chap. 12 by Bushby**). In tendons, when collagen VI is removed via null *Col6a1* mouse model, tenocyte expression changes due to a lack of cell-matrix interactions [42]. In tendons, the absence of collagen VI results in increases in fibril density, significant reductions in load and stiffness, increased matrix metalloproteinase activity, and overall dysfunctional regulation of fibrillogenesis [42].

Collagen VI is commonly formed as a heterotrimer composed of α1(VI), α2(VI) and α3(VI) chains [39, 44]. Each monomer has a 105 nm triple helical domain with flanking N- and C-terminal globular domains. The α3(VI) chain of the heterotrimer can be processed extracellularly. In addition, structural heterogeneity is introduced by alternative splicing of domains, primarily of the α3(VI) N–terminal domain. Three additional α chains of type VI collagen have been described, α4(VI), α5(VI), α6(VI); these chains have high homology with the α3(VI) chain and may form

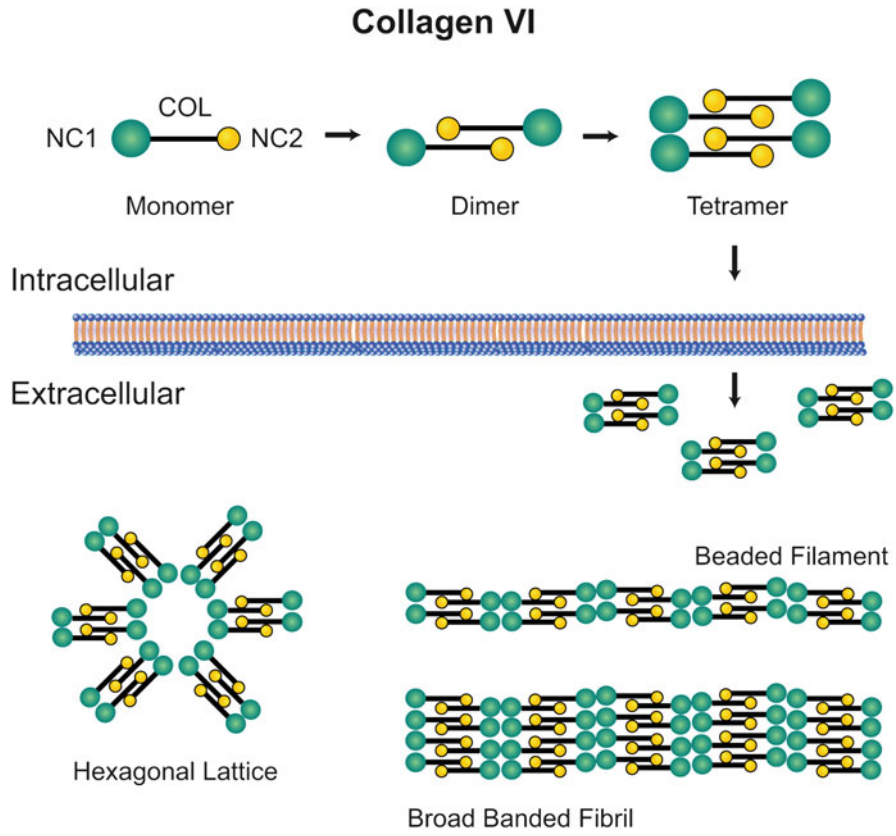

Fig. 2.4 Assembly of collagen VI suprastructures. Collagen VI monomers have a C-terminal NC domain, a central triple helical domain, and an N-terminal NC domain. The monomers assemble N-C to form dimers. Tetramers assemble from two dimers aligned in-register. The tetramers are secreted and form the building blocks of 3 different collagen VI suprastructures: beaded filaments, broad banded fibrils and hexagonal lattices. These suprastructures form via end-to-end interactions of tetramers and varying degrees of lateral association (This figure has been adapted from Birk and Bruckner [131])

additional isoforms [45, 46]. The supramolecular assembly of collagen VI begins intracellularly (Fig. 2.4). Two collagen VI monomers assemble in a lateral, anti-parallel fashion to form a dimer; the monomers are staggered by 30 nm with the C-terminal domains interacting with the helical domains. The resulting overlap generates a central 75 nm helical domain flanked by a non-overlapped region with the N– and C-globular domains, each about 30 nm. The C-terminal domain-helical domain interactions are stabilized by disulfide bonds near the ends of each overlapped region [47]. The overlapped helices form into a supercoil of the two monomers in the central region [48]. Two dimers then align to form, tetramers, also intracellularly. The tetramers are secreted and associate end-to-end to form beaded filaments extracellularly. The newly formed thin, beaded filaments (3–10 nm) have a periodicity of approximately 100 nm; they laterally associate to form beaded microfibrils [36], and they are found in hexagonal lattices [49]. The broad banded fibrils represent continued lateral growth of beaded microfibrils and/or lateral association of preformed beaded microfibrils. In contrast, hexagonal lattices are formed via end-to-end interactions of tetramers in a non-linear fashion [49].

Like fibrillar collagen structures, collagen VI-rich supramolecular aggregates are composite structures with other integrated molecules that

modulate the functional properties of the ultimate suprastructure. For example, biglycan interactions with the collagen VI tetramer induced formation of hexagonal lattices, instead of beaded microfibrils; though, decorin, which binds to the same site, was less effective in inducing hexagonal lattice formation [49]. Thus, analogous to fibril formation, the interaction of small leucine-rich proteoglycans with collagen VI can influence the structure of the tissue aggregate and therefore its function. The interaction of collagens with such molecules in a multitude of ways allows for the assembly of different suprastructures in adjacent regions or tissues with different functions, even sometimes as simply as with the addition or removal of non-collagenous molecules.

2.7 Network-Forming Collagens

Collagens VIII and X are closely related short chain collagens, with comparable gene and protein structures [39, 50]. While collagen VIII is not typically found in musculoskeletal tissues, collagen X has a very restricted distribution, found only in hypertrophic cartilage. This collagen is a homotrimer composed of α1(X) chains and the supramolecular form is a hexagonal lattice [51]. Collagen X was recently reviewed [52].

2.8 Transmembrane Collagens

Transmembrane collagens include collagens XIII, XVII, XXIII, and XXV. They are all homotrimers and contain an N-terminal cytoplasmic domain and a large C-terminal domain containing multiple COL domains with NC interruptions providing flexibility. Transmembrane collagens are so classified because they have a hydrophobic membrane spanning domain. Between this domain and an adjacent extracellular linker domain is the first COL domain involved in trimerization which is also subject to proteolytic cleavage generating a shed extracellular domain. Of the transmembrane collagens, collagen XIII is found in musculoskeletal tissues, particularly in myotendinous and neuromuscular junctions [53, 54].

2.9 Procollagen Synthesis, Collagen Fibril Assembly, Growth and Maturation

Collagen synthesis, assembly, and maturation require a sequence of well controlled intracellular and extracellular events. Collagen genes are transcribed from DNA into mRNA within the nucleus. Transcripts are transported out of the nucleus and translated into procollagen monomers, which are post-translationally modified in the rough endoplasmic reticulum prior to assembly into procollagen triple helices. The extent of these modifications is affected by the rate of triple helix formation, which is in turn affected by the primary structure of the alpha chain propeptides. For example, point mutations in the Gly-X-Y sequence may result in altered molecular properties for that chain as well as dysfunctional regulation of chain selection, helix formation, or post-translational modification. These molecules are then secreted as procollagens, which prevents premature molecular assembly into suprastructures. Procollagens are then processed extracellularly, in most cases, and require collagen type-specific metalloproteinases. Processing may, however, begin during the transport of newly synthesized procollagens to the cell surface [55, 56]. Processed collagen triples helices are then cross-linked. The relationship between protein structure and triple helix assembly and collagen fibril formation will be discussed below, as well as the growth and maturation of fibrils in tendons and ligaments.

2.10 Triple Helix Assembly and the Impact of Primary Structure on Secondary, Tertiary, and Quaternary Structures

After collagen pre-pro-peptides have been translated, they are directed into the lumen of the rough endoplasmic reticulum (RER). Modifications in the pre-pro-peptide include cleavage of the N-terminal signal for transport to the RER so that

it becomes a propeptide, or a pro-alpha chain [57]. Pro-alpha chains undergo hydroxylation of prolyl and lysyl residues followed by glycosylation of hydroxylysyl residues. Three pro-alpha chains will trimerize; this involves the selection and alignment of appropriate alpha chains with subsequent assembly into specific trimeric collagen molecules [58]. Trimerization is initiated through interactions among the non-collagenous trimerization domains of alpha chains at their C-termini in the RER [57].

The primary amino acid sequence of collagen alpha chains affects the protein structure at all other levels. The primary structure of the collagen alpha chain features a COL domain that will coil into a left-handed helix lacking intra-chain hydrogen bonds. Three alpha chains will supercoil to form a triple helix. This parallel, right-handed superhelix is stabilized by inter-chain hydrogen bonds that are almost perpendicular to the triple helical axis. Because of the imino acid content, collagen polypeptides assume an elongated polyproline II-like helix with all peptide bonds in the *trans* configuration. The pitch of the polyproline II helix in collagenous polypeptides corresponds to three amino acids, almost exactly. Steric constraints require that only glycine, the smallest amino acid, can occupy the positions at the center of the triple helix. Thus, the triple helical domains have a repeating $(Gly\text{-}X\text{-}Y)_n$ structure with the X and Y positions being any of the 21 proteinogenic amino acids of the universal genetic code. However, the X and Y positions are typically occupied by proline and hydroxyproline, which are necessary for helix formation and stability, respectively. Replacement of the glycine residues with other amino acids interrupts the triple helix motif and causes the rod-like structures to have rigid kinks or flexible hinges [59, 60]. This occurs in many collagen types with primary structures containing more than one helical domain thus providing flexibility, e.g., in collagen V. However, the substitution of glycine residues also may be the result of missense mutations in collagen genes and manifest as the underlying etiology for mild or severe, systemic connective tissue diseases [61, 62].

As post-translational modification and triple helix assembly occur concomitantly, once initiated, trimerization must be controlled to allow for alpha chain post-translational events like hydroxylation and glycosylation to occur. After or as collagen alpha chains are translated, amino acids within the triple helical domains of each chain can be modified. Co-translational modification of these domains includes hydroxylation and glycosylation. Collagen polypeptides contain two unique amino acids, hydroxyproline and hydroxylysine, which are important downstream for triple helix stability and glycosylation, respectively. As the propeptides are synthesized, these unique amino acids are introduced by enzymatic hydroxylation of almost all prolyl- and some of the lysyl residues in the Y-positions. Hydroxyproline residues are important in triple helix stability. The 4-trans hydroxyl group assists in directing the free hydroxyproline, similar to that of Y-hydroxyproline in collagen triple helices. The forced integration of proline into Y-positions absorbs more ring deformation when compared with physiological hydroxylated collagen [63, 64]. Collagens are further modified post-translationally after secretion and during aggregate assembly.

Fibril structure also is affected by post-translational glycosylation of collagens. Covalent modifications occurring after polypeptide synthesis are important in collagens and have an impact on fibril assembly [65–67]. The circumference of collagen triple helical domains is affected by the extent of hydroxylation of lysyl residues and subsequent galactosylation and glucosyl-galactosylation of hydroxylysyl residues. Intermolecular center-to-center distances correlate with the extent of glycosylation, particularly if post-translational modifications affect those regions of polypeptides situated in overlap regions of the fibril. The extent of glycosylation of hydroxylysine modifications can be manipulated by features such as increased enzyme activity levels[68] and disease-causing mutations [62]. Rates of procollagen triple helix formation can be substantially reduced in the rough endoplasmic reticulum by collagen mutations. Over-modification leads to compromised fibrillar organization. Yet

different extents of glycosylation can serve as a physiological mode of regulation in normal native tissue, particularly when levels of post-translational modification are tissue-specific and thus contribute to the structural properties of tissues. Intrafibrillar water also has been shown to affect the molecular organization of collagen fibrils. As variable amounts of water are incorporated within the fibrils, intermolecular distances between lateral or longitudinal neighbors have been shown to differ [69, 70]. When collagen fibrils are dried, their D-periodicity shortens intermolecular lateral distances reduce.

Collagenous domains of alpha chains are distinctly rich in *cis*-peptide bonds due to their high content of imino acids that favor *cis*-peptide bond formation. Kinetically, a great deal of energy is required to cause *cis*-peptide bonds to comply with triple helix formation. Isomerization of each *cis*-peptide bond encountered is necessary during collagen triple helix formation which is a zipper-like process. Helix assembly thus involves a slow folding process when compared to other proteins [71, 72]. At the start of triple helix formation, a variable number of *cis* bonds are distributed throughout the still unfolded procollagen polypeptides. Hence, the folding times required for full-length triple helix formation are heterogeneous depending upon kinetic obstacles. In fibroblasts, the *cis* to *trans* isomerization of Gly-Pro-, but not X-Hyp peptide bonds, is catalyzed by cyclophilin B, which acts as peptidyl prolyl *cis/trans*-isomerase. Cyclophilin B can be inhibited by the immuno-suppressor cyclosporin A [73]. Moreover, cyclophilin B along with prolyl-3-hydroxylase and cartilage-associated protein (Crtap) form a ternary complex with high chaperone activity in the endoplasmic reticulum. Prolyl-3-hydroxylase introduces a single 3-Hyp-residue at the C-terminal end of the triple helical domain of nascent fibrillar procollagens. This ternary complex localizes cyclophilin B-activity to the initiation sites for procollagen folding. This allows for efficient catalytic isomerization of peptidyl-prolyl *cis* bonds. Such a role is supported by data from humans where null mutations in LEPRE1 and CRTAP, the genes encoding human prolyl-3-hydroxylase and Crtap, respectively, cause severe recessive osteogenesis imperfecta [74].

Additional modifications occur post-translati onally after secretion of procollagen and during supramolecular assembly. Extracellular lysyl oxidases can convert the amino groups on some of the hydroxylysine- and lysine residues in the collagen polypeptide chain to aldehydes that form aldols or β-ketoamines [57]. This happens when the aldehydes of hydoxylysine or lysine residues react with aldehydes or amino groups on lysines, respectively, in other chains to generate intra- and inter-molecular covalent crosslinks [75–77]. In some cases, cross-linking of collagen molecules at early stages of aggregation can modulate the suprastructural outcome of fibrillogenesis or the formation of networks.

2.11 Collagen Fibril Assembly

The assembly and deposition of collagen fibrils with tissue-specific structures and organizations involves a sequence of events that occur in both intracellular and extracellular compartments. Collagen molecules are synthesized, hydroxylated, glycosylated, assembled from three polypeptides, and folded in the rough endoplasmic reticulum. Then packaging of the trimers occurs in the Golgi, and transport is via specialized and elongated intracellular compartments with secretion at the cell surface. Collagen fibrils are composites of different matrix molecules, and control of heteropolymeric mixing and trimer type stoichiometry begins within the intracellular compartments. Moreover, secretion of different matrix molecules and modifying enzymes occurs with specific spatial, temporal, and circumstantial patterns. Therefore, the character of the assembled fibrillar matrix depends upon not only the collagen types synthesized, but on the regulated interactions with procollagen processing enzymes, fibril-associated molecules (e.g., proteoglycans and FACITs), and adhesive glycoproteins. The spatial and temporal regulation of mixing during packaging and transport or at the sites of secretion provides a mechanism whereby limited numbers of matrix molecules can be

assembled to produce the diversity of structure and function observed across tissues.

Extracellularly in the developing tendon, fibril assembly begins in deep recesses or channels defined by the fibroblast surface [55, 78–80]. In these micro-domains, protofibrils are assembled [78, 81]. These immature fibrils have small and uniform diameters as well as short lengths compared to mature fibrils. These extracellular channels have been shown to form at the time of secretion as specialized post-Golgi secretory compartments, fuse with the fibroblast membrane and are maintained due to slow membrane recycling associated with the presence of the assembled protofibril [78, 81]. Other data also suggest that intracellular processing of procollagen may occur within elongated Golgi-to-plasma membrane compartments (GPCs); this is followed by the extrusion of protofibrils through a cellular protrusion, where GPC fuse to fibroblast plasma membranes [55, 79]. Either way, protofibrils are ultimately present extracellularly. Once the protofibrils are deposited into the extracellular matrix, more compartmentalization occurs. That is, fibrils form small fibers and then larger structures characteristic of the specific tissue (e.g., large fibers in tendon). This compartmentalized hierarchy within the extracellular allows the fibroblast to exert control over the extracellular steps of matrix assembly (Fig. 2.5).

2.12 Assembly and Growth of Mature Tendon Collagen Fibrils

In mature tendon, collagen fibrils are functionally continuous. That is, fibrils are long, though lengths unmeasured, and have diameters ranging between 20 and 500 nm depending on the tissue and developmental stage [82–84]. However, during tendon development collagen fibrils are assembled near the fibroblast surface as uniform and relatively short D periodic protofibrils with diameters in the range of 20–40 nm, lengths of 4–12 μm, and tapered ends [81, 82, 85, 86]. The newly assembled protofibrils are deposited and incorporated into the developing tendon extracellular matrix as small bundles of fibrils or immature fibers. There they are stabilized via interactions with macromolecules such as FACITs and small leucine-rich proteoglycans (Fig. 2.6). Tendon maturation continues with linear fibril growth involving end overlap of the protofibrils, followed by lateral growth in tendons and ligaments. Lateral fibril growth features the association and fusion of fibrils laterally to generate larger diameter fibrils. This process involves molecular rearrangement of fibrils so that a cylindrical fibril structure is generated. To accomplish this, some or all of the collagen stabilizing components stabilizing are lost or replaced. Throughout this process, the number of intra- and intermolecular crosslinking plays an important role in regulation of structure turnover and stability. Increases in stability generated by crosslinking improve mechanical stability that varies by location within tendons and ligament and anatomically throughout the body. The specific roles of collagen accretion in fibril assembly/repair during regeneration remain to be elucidated. Moreover, contributions in tissue homeostasis and with normal turnover and repair are likely.

2.13 Regulation of Collagen Fibril Assembly and Growth

Regulation of collagen fibrillogenesis is tissue-specific. Within each tissue, interactions occur amongst many different classes of molecules, such as processing enzymes, heterotypic fibril-forming collagens, FACITs, and small leucine-rich proteoglycans (SLRPs) as well as other glycoproteins, such as, fibronectin, tenasin X, etc. Many regulatory interactions occur and one way to impose some order on the discussion is to consider three classes of interactions; molecules that act as organizers, nucleators, and regulators [87].

For example, significant increases in collagen fibril diameters occur during growth and development of tendon, and mechanical properties of the tendon are dependent on increases in fibril diameter seen.[88] Tendon fibroblasts express collagen I as the major fibril-forming collagen with minor

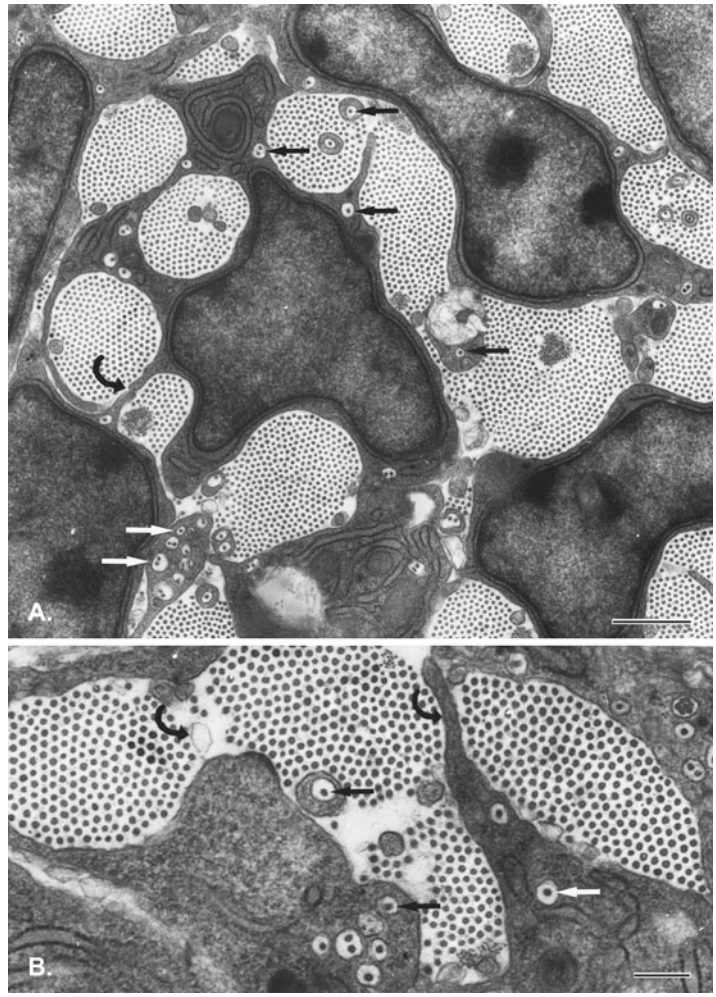

Fig. 2.5 Extracellular compartmentalization of fibril and fiber assembly by tenocytes. In the developing chicken tendon, extracellular compartmentalization of the different levels of matrix assembly is seen in both panels (**a**) and (**b**). These panels were generated from cross-sections of 14-day chicken embryo tendon cut perpendicular to the tendon axis A series of micro-domains are evident. Protofibrils are assembled in fibril-forming channels (*arrowheads*). Protofibrils are deposited into the developing matrix in fiber-forming spaces where fibers coalesce to form fibers. Fibers continue to become larger as a result of the aggregation of adjacent fibers in a third domain. As cytoplasmic processes that define fiber-forming compartments retract (*curved arrow*), fibers (fibril bundles) are allowed to coalesce into larger aggregates characteristic of mature tissue. Bar, 1 micrometer (Modified from Birk and Linsenmayer 1994)

quantities of collagen V. Thus, tendon fibrils are heterotypic, containing collagens I and V.

Collagens V and XI have been shown to nucleate collagen fibril formation in self-assembly assays [89–92]. The alpha-chains of collagens V and XI are highly homologous; thus, these collagens represent different isoforms of a single collagen type. A mouse model with a targeted deletion within the *Col5a1* gene is embryonic lethal due to a lack of fibril formation in the mesenchyme; though collagen I is synthesized and secreted, protofibrils are not properly assembled [91]. Similarly, in two separate mouse models where collagen XI is absent (cho/cho, ablated *Col11a1* alleles; *Col2a1*-null mice, from which the α3(XI) chain is derived), animals develop chondrodysplasia (cho) where cartilage is devoid of fibrils.[93–96] These data demonstrate a key

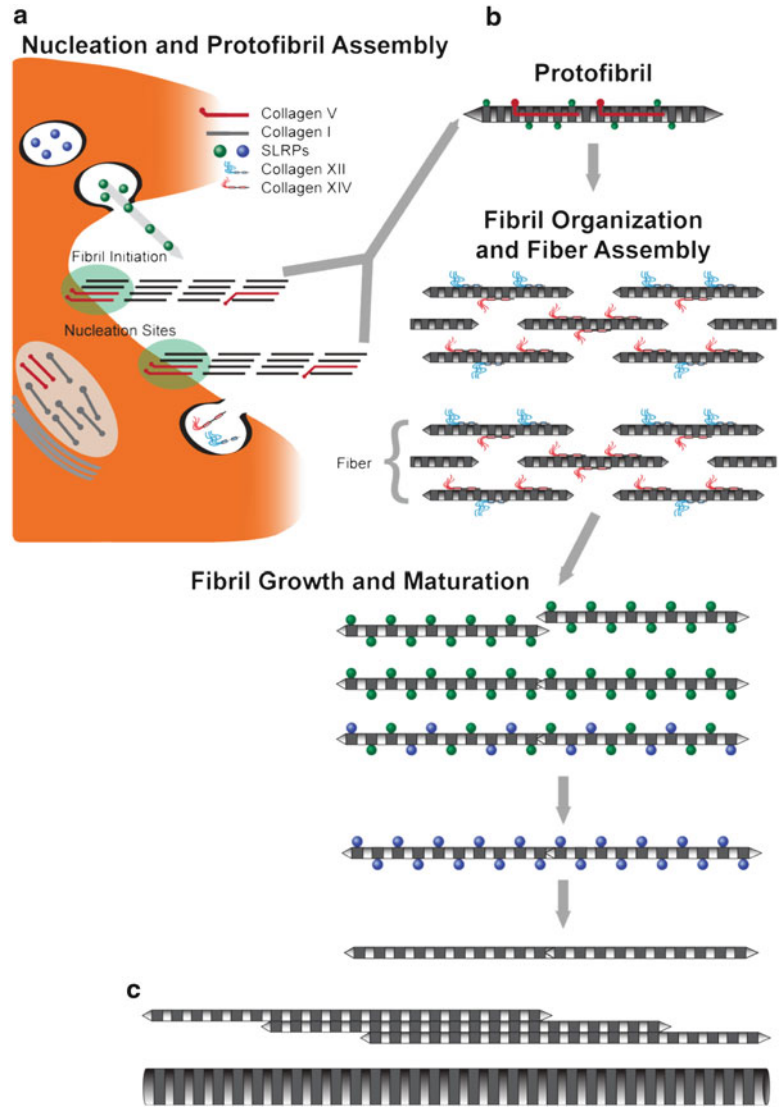

Fig. 2.6 Model of the regulation of fibril assembly. Model of the regulation of fibril assembly. Fibril assembly involves a sequence of events. (**a**) First, nucleators (e.g., collagen V and XI) initiate fibril assembly at the fibroblast cell surface. Then immature, small diameter, short protofibrils are assembled. The nucleation process is cell directed involving interactions with organizers (e.g., integrins and syndecans) at the cell surface. (**b**) Protofibrils are deposited into the matrix and are stabilized by interactions with regulators – other matrix components such as SLRPs and FACITs. (**c**) Changes in fibril stabilization resulting from processing, turnover and/or displacement regulate linear and lateral growth to mature fibrils in a tissue-specific manner (This figure has been adapted from Birk and Bruckner [131])

role for collagen V/XI in nucleation of assembly of short, small diameter protofibrils.

Under physiological conditions in vitro, collagens I and II can self-assemble after long lag phases. The nucleation of fibril formation by collagen V or XI provides a mechanism for the fibroblast to define the site of fibril formation. That is, the number of originating fibrils is controlled by the number of nucleation sites provided by the fibroblast. This feature is tissue-specific. For example, in the cornea where smaller fibril diameters are paramount to transparency,

collagen V is 10–20 % of the fibril-forming collagen content, and the many nucleation sites contribute to many smaller diameter fibrils [97, 98]. However, in tendons where mechanical strength depends upon larger diameter fibrils, collagen V makes up 1–5 % of the fibril-forming collagen content. One classic example of the collagen I/V interaction and its impact on structure is best seen in the classic form of Ehlers-Danlos Syndrome, a generalized connective tissue disorder where the majority of patients are heterozygous for mutations in collagen V [99]. This disorder results in approximately 50 % of the normal collagen V. Affected patients have a dermal phenotype with large, structurally aberrant fibrils. Similarly, in the heterozygous (*Col5a1+/−*) mouse model, there was a reduction of 50 % in collagen V fewer fibrils assembled, indicating fewer nucleation events than the normal mice [91], a similar situation occurs in the tendon [21]. The reduction in nucleation sites makes collagen V a rate limiting molecule in this instance. The regulation of these collagen I/V interactions is coordinated by the domain structure at the sites of assembly and by other molecules that organize and sequester these interactions at the cell surface [9].

The nucleation of protofibrils involves direction from cellular structures such as organelles, cytoskeletal components, cytoplasmic membrane domains, as well as organizing molecules. However, equally important are cell-defined extracellular domains. Without such direction, fibril assembly is inefficient. For example, in monolayer cultures, in the absence of extracellular matrix, procollagens are secreted freely into the media. Thus, organizing molecules provide a resource for tissue-specific coupling of fibril assembly to the cell surface. Consequently, cell-directed positioning of the deposited matrix is possible as assembled protofibrils undock from the cell surface, are extruded from the cell, and are incorporated into the extracellular matrix so that nucleation sites might be re-primed for the next round of nucleation.

Cell-directed collagen fibril assembly involves organizing molecules such as integrins and fibronectin. Fibronectin mediates the cell's interaction with assembling collagen fibrils as well as with other extracellular matrix molecules. Fibronectin molecules assemble into fibrils via integrin interactions [87, 100, 101]. The fibronectin fibril network contains multiple binding sites for collagen fibril assembly. When binding sites are blocked in fibronectin networks, collagen fibril assembly is inhibited [102]. Moreover, modifications to fibronectin-integrin interaction affect collagen fibril assembly, suggesting both that the cytoskeleton is involved in some way with collagen fibril assembly and that other direct interactions of integrins with other surface molecules are essential to fibril assembly.

Once the protofibrils are assembled and deposited into the extracellular matrix, further assembly to the mature fibrils involves linear and lateral growth of the preformed intermediates. In tendons and ligaments, numerous molecules are involved in the regulation of these steps. Two classes of regulatory molecules are the small leucine-rich proteoglycans (SLRPs) and the FACIT collagens. Both classes are fibril-associated and molecules within each class have their own tissue-specific, temporal, and spatial expression patterns. Differences in expression patterns contribute to the differences in structure and function amongst tissues, including tendons and ligaments.

Small leucine rich proteoglycans (SLRPs) are important regulators of linear and lateral fibril growth [103]. Two classes of SLRPs are expressed throughout tendon growth and maturation. They are divided into class I (decorin and biglycan) and class II (fibromodulin and lumican). When the genes for these molecules are specifically targeted via single or compound SLRP deficient mice, their importance in regulation of linear and lateral fibril growth in tendons is demonstrated [103–109]. In tendons, decorin and fibromodulin are dominant in this regulation, and they can be modulated by biglycan and lumican, respectively [88, 110]. A lack of decorin, biglycan, or fibromodulin leads to disruptions in fibril growth, resulting in larger diameter fibers, structural abnormalities, and biomechanical alterations in the tendon [88, 104, 111–114]. Moreover, synergistic (additive) effects are seen between classes when compound biglycan and

fibromodulin deficiencies occur that affect fibril diameters, alter tendon biomechanics, and even promote ectopic ossification within the tendon [104, 111, 115]. Unlike fibril-forming collagens, SLRPs regularly turnover and more easily allow for changes in expression to affect fibrillogenesis and tendon structure throughout development, maturation, and injury. A more detailed description of activities mediated by SLRPs can be found in Chap. 4.

The regulation of linear and lateral fibril growth is also affected by FACIT collagens. As described earlier in the chapter, FACIT collagens are fibril-associated molecules with large non-collagenous domains. Like SLRPs, FACITs also demonstrate differing tissue-specific and temporal expression patterns. Collagen IX is involved in regulation of fibril growth in cartilage [23]. Collagen XIV has recently been implicated in the regulation of tendon fibril growth [25]. Targeted deletion of collagen XIV in mouse models demonstrated a premature entrance into the fibril growth stage in tendons, resulting in larger diameter fibrils in early developmental stages. This indicates that in some tissues like tendons FACITs may serve as 'gate keepers' regulating the transition from protofibril assembly to fibril growth during development. In the case of collagen XIV, it temporarily stabilizes protofibrils to prevent the initiation of lateral fibril growth. The large non-collagenous domain and its inter-fibrillar location have been long suspected to play an additional role in fibril packing. Control of fibril packing also would influence lateral associations necessary for growth.

2.14 Effects of Composition and Structure on Function for Tendons and Ligaments

Tendons and ligaments are dense connective tissues composed of similar proteins and other macromolecules. Both tissues have a large extracellular matrix to cell ratio. However, slight differences in content and morphology allow for each tissue type to function in a distinct manner. The composition of the extracellular matrix in tendon is crucial for transmitting the force that is generated from muscle to bone. In contrast, the ligament has a more passive role of attaching bone to bone, guiding the joints motion, and preventing abnormal displacement of bones; to a minor extent, ligaments must also withstand load to resist joint instability. Collagens make up 60–70 % and 70–80 % of the dry weight for ligaments and tendons, respectively [1, 116]. The predominant fibril-forming collagen of tendons and ligaments is collagen I; other collagens within tendons and ligaments include III, V, VI, XII, and XIV. Besides collagens, tendons and ligaments contain many of the macromolecules mentioned throughout this chapter that regulate fibril assembly as well as water (60–70 % in ligaments and 50–60 % in tendons) [116]. Thus, while the content of these tissues is quite similar, there are distinctions in proportional compositions of water and macromolecules. Likewise, there are several similarities in the morphologies of the two tissues. Both tendons and ligaments have many parallel fibers that run along the axis of tension and these fibers are composed of mainly fibrils containing collagen I. There is also a "crimping" pattern in the collagen fibers of both tissues. However, there are several morphological distinctions. For example, within ligaments there are many regions where collagen fibers intertwine with adjacent fibers so that subsets of fibers are running obliquely at a 20–30° angle from the other fibers running along one axis [117]. These differences in composition and morphology are enough to contribute to the function of the tissues.

Composition of both tendons and ligaments contributes to the nonlinear anisotropic mechanical behavior that is exhibited in both tissues. An example load-elongation curve is depicted in Fig. 2.7. When loading conditions are low, tendons and ligaments are relatively compliant. In the "toe" region of the curve, initial load is affecting the "crimped" collagen fibers and any viscoelastic properties provided by molecules interacting with the collagens. The "toe" regions of the tendon is smaller because most collagen fibers are oriented parallel to the directions of strain and thus less realignment is necessary. With increasing

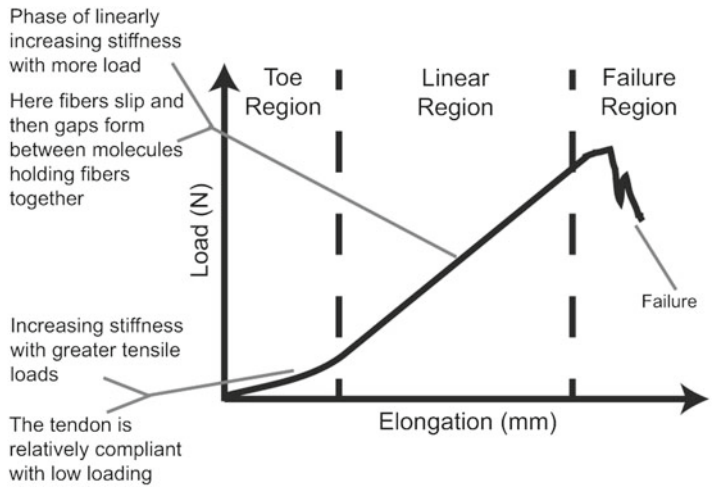

Fig. 2.7 Load-elongation curve for tendons and ligaments. There are three distinct regions within the curve which define the response to tensile loading. In the "Toe Region," initial load is affecting the "crimped" collagen fibers and any viscoelastic properties provided by molecules interacting with the collagens. In the "Linear Region," increasing tensile loads cause lengthening as the tendon or ligament becomes increasingly stiffer. In this region, load/elongation will ultimately follow a linear slope as slippage occurs within and then between collagen fibrils. In the "Failure Region," load continues to increase until the point of tearing as adjacent fibril molecules slip away to tensile failure, or complete rupture

tensile loads, these tissues – tendons more so than ligaments – become increasingly stiffer. At some point, load/length, or stiffness, will follow a linear slope as slippage occurs within collagen fibrils, next between fibrils, and then until the point of tearing as adjacent fibril molecules slip away with tensile failure (termed, ultimate load or point of ultimate tensile stress). As the load proceeds from initial strain to the point of failure, the area under the curve is considered the total energy absorbed.

Though both tendons and ligaments are considered to exhibit a nonlinear anisotropic mechanical behavior, differences in their load-elongation curves do exist. As already mentioned, the morphology of the ligament allows for the "toe" of the curve from the initial loading to be longer because the crimping pattern of the ligament is more greatly affected by fibrils not oriented exactly parallel – sometimes even perpendicular to the direction of the load. The stiffness of tendons and ligaments are also distinct. In humans, the elastic modulus of a tendon (Achilles tendon, 375 ± 102 MPa; biceps tendon, 421 ± 212 MPa; patellar tendon, 660 ± 266 MPa) is generally greater than that of a ligament (meniscofemoral ligament, 355 ± 234 MPa; anterolateral bundle of the posterior cruciate ligament, 294 ± 115 MPa; posterior bundle of the posterior cruciate ligament, 150 ± 69 MPa) [118, 119]. Moreover the stiffness for tendons (Achilles tendon 430 N/mm) is generally greater than for ligaments (lateral/medial collateral ligaments, 20 N/mm; anterior cruciate ligament, ACL 182 N/mm) [120–122]. Greater values for elastic modulus and stiffness are indicative of stiffer, less flexible connective tissue that is capable of absorbing and transmitting more energy. In addition, the tensile strength of tendons (50–150 N/m^2) is greater than that of ligaments (26–39 N/m^2) [123–125]. The compositional differences and distinctions in stiffness and tensile strength between tendons and ligaments all contribute to the understanding of how these two connective tissues function.

Tendons and ligaments have different roles. Tendons center the actions of several muscles into one axis of stress or strain. They distribute contractile force of muscles to bones, and they provide the muscle with distance from the insertion

that might mechanically beneficial. Tendons store elastic energy during locomotion, and prevent muscle injury with viscoelasticity. The relative stiffness and tensile strength of tendons is essential for maintaining force transmission. Ligaments, on the other hand, guide joint motion by attaching adjacent bones involved. This stabilizes the joint and controls the range of motion when load is applied. Their flexibility, relative to tendons, allows for range of motion within the joint. Tendons are susceptible to injury from overuse, wear and tear, and abrupt tears or avulsions when great forces are applied. Ligaments, though flexible, have less tensile strength and are prone to shear force injuries. The functions of tendons and ligaments are made manifest by thorough consideration of their composition, morphology, and physiology.

While tendons and ligaments possess characteristics that might distinguish one connective tissue from another, each of these tissue types also differs by anatomical location. For example, features of an Achilles tendon are not identical to those of a flexor digitorus profundus tendon or a patellar tendon. Likewise, characteristics of an anterior cruciate ligament are not identical to those of a medial collateral ligament. In addition, within each tendon and ligament, there are zones where composition changes. For example, ligaments can be divided into ligament mid-substance, fibrocartilage, mineralized fibrocartilage, and bone. Similarly, tendons have musculotendinous junctions, mid-substance, fibrocartilage, and mineralized fibrocartilage to the enthesis. In the following sections, differences in collagen structure and physiology will be described by anatomic location and by zone.

2.15 Effect of Anatomical Location on Tendons and Ligaments

While most tendons and ligaments are generally composed of the same content described throughout the chapter, there are slight differences in gross structure and content that might better allow the tendon to function at its anatomical location [1]. Tendons like that of the flexor digitorus profundus are round and contain the typical parallel bundles of collagen fibers. However, tendons such as those in the rotator cuff are flat, layered, and multi-directional; each tendon contains parallel collagen fibers as well as fibers that interdigitate obliquely with fibers from other tendons within the rotator cuff [126]. Rotator cuff tendons also contain more proteoglycans throughout than the typical round tendons; additional proteoglycans are believed to be aggrecan and SLRP biglycan [127]. A study of ovine tendons and ligaments demonstrated in an extracellular matrix analysis that each tendon (long digital extensor tendon, superficial digital flexor tendon, patellar tendon) and ligament (lateral collateral ligament, medial collateral ligament, posterior cruciate ligament, anterior cruciate ligament) had its own unique range for matrix compositions when examining water, glycosaminoglycan, and collagen content [116]. Moreover, each tendon and ligament demonstrates its own collagen organizational and mechanical features [116]. Gross anatomical differences in tendon and ligament size and shape occur as these connective tissues: traverse areas with limited space (e.g., within the wrist), centralize the force of several muscles (e.g., the Achilles tendon), or manage multi-directional forces and movements by intertwining collagen fibers with fibers of nearby connective tissues associated with tension in another axis (e.g., cruciate ligaments and rotator cuff tendons). The precise composition and structural arrangement of each tendon and ligament provides specific mechanical properties to allow that connective tissue to function. Thus, to some extent, each tendon and ligament has its own unique features.

2.16 Roles of Collagens in Transition from Midsubstance to Enthesis in Tendons and Ligaments

Much of what has been described in this chapter to this point has covered the tendon and ligament mid-substance. The transition of tendons

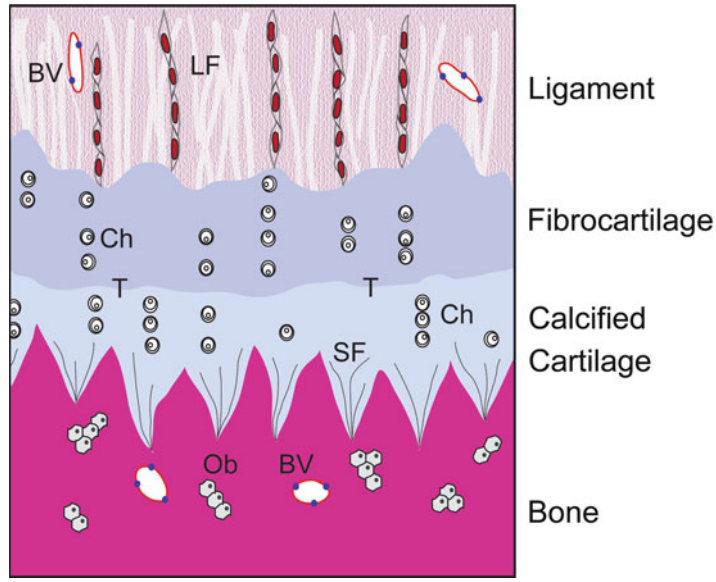

Fig. 2.8 Transition zones. This illustration demonstrates the transition from fibrous tissue to bone at a ligament insertion. The tissue transitions from ligament to fibrocartilage. Increases in the level of calcification are noted in the compositional gradient closer to insertion; this compositional change is demarcated by a tidemark (*T*) which traces the interface between non-calcified fibrocartilage and the next zone, calcified fibrocartilage. Calcified fibrocartilage interdigitates with the underlying subchondral bone to complete the insertion. Structures of note within the illustration include: ligament fibroblasts (LF), fibrocartilage chondrocytes (Ch), osteoblasts (Ob), Sharpey's fibers (SF), and blood vessels (BV) (This figure has been adapted from Place et al. [132])

and ligaments toward entheses are associated with changes in their composition and organization. There is a transition from tendinous and ligamentous material to bone (Fig. 2.8). These transition sites are not simple discreet units; instead, there is more of a gradient of molecular differences from mid-substance to bone. That said, one can identify general zones: mid-substance, fibrocartilage, calcified fibrocartilage, and bone [128]. The first zone consists of mid-substance or tendon proper; its composition has been described throughout the chapter. Basically, this zone contains collagen I-rich fibers that are aligned parallel to one another along the axis of strain, as well as a small amount of collagens V, VI, XII, and XIV, decorin, and other matrix macromolecules. The second zone is best characterized as fibrocartilage. It is predominantly composed of collagens II and III with minor amounts of collagens I, IX, and X, as well as aggrecan and decorin [129]. The composition of the second zone departs from that of the tendon or ligament-midsubstance. The third zone contains mineralized or calcified fibrocartilage. It is primarily composed of collagen II with significant amounts of collagen X and aggrecan [130]. The fourth zone is characterized as bone; it is predominantly composed of collagen I as well as components typically found within bone. The continuity of the enthesis is an efficient way to transfer and buffer loads applied between muscle, tendon, and bone or from bone to bone. The structure and composition of the region from mid-substance to fibrocartilage accommodates loads along the axis of the tendon, while the region with mineralized fibrocartilage and bone zones manages complex multidirectional forces that occur nearer to the bone [130]. The gradation of this transitional structure is difficult to replicate in surgery with native of engineered grafts.

2.17 Summary

The functions of tendons and ligaments depend greatly upon their extracellular matrices, particularly the collagen that comprises 70–80 % of their dry weight. Collagens found within tendons and ligaments belong to several subfamilies that can be grouped by their predominant suprastructural forms; these include: fibril-forming, FACIT, basement membrane, and beaded filament-forming collagens. The many collagens provide considerable diversity for the functional extracellular matrix suprastructures. This diversity is further compounded by the numbers of different alpha chains formed; by alternative splicing; as well as by the many types of post-translational modifications. Overall it is the suprastructural organization of these collagen molecules that provides tissue-specific structure and function, particularly to tendons and ligaments. These suprastructures are macromolecular heteropolymers containing different collagens and other fibril-associated molecules. Within tendons and ligaments, the assembly of these collagen suprastructures relies upon the primary structures of these molecules that contain domains that will affect downstream secondary, tertiary, and quaternary structures. Clearly the content and organization of these connective tissues affect how they function in absorbing and transmitting forces as well in maintaining stability. While generalizations can be made for both connective tissues, current studies demonstrate that differences exist between different tendons as well as between tendons and ligaments. Each tissue's basic composition and structure will affect its unique physiology. Thus, while an understanding of how collagen is assembled and organized is critical for tendon and ligament repair and regeneration, it is also essential to focus on how these general mechanisms generate unique structures that will determine each tissue's distinct features and functional properties.

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3

Basic Components of Connective Tissues and Extracellular Matrix: Elastin, Fibrillin, Fibulins, Fibrinogen, Fibronectin, Laminin, Tenascins and Thrombospondins

Jaroslava Halper and Michael Kjaer

Abstract

Collagens are the most abundant components of the extracellular matrix and many types of soft tissues. Elastin is another major component of certain soft tissues, such as arterial walls and ligaments. Many other molecules, though lower in quantity, function as essential components of the extracellular matrix in soft tissues. Some of these are reviewed in this chapter. Besides their basic structure, biochemistry and physiology, their roles in disorders of soft tissues are discussed only briefly as most chapters in this volume deal with relevant individual compounds. Fibronectin with its muldomain structure plays a role of "master organizer" in matrix assembly as it forms a bridge between cell surface receptors, e.g., integrins, and compounds such collagen, proteoglycans and other focal adhesion molecules. It also plays an essential role in the assembly of fibrillin-1 into a structured network. Laminins contribute to the structure of the extracellular matrix (ECM) and modulate cellular functions such as adhesion, differentiation, migration, stability of phenotype, and resistance towards apoptosis. Though the primary role of fibrinogen is in clot formation, after conversion to fibrin by thrombin, it also binds to a variety of compounds, particularly to various growth factors, and as such fibrinogen is a player in cardiovascular and extracellular matrix physiology. Elastin, an insoluble polymer of the monomeric soluble precursor tropoelastin, is the main component of elastic fibers in matrix tissue where it provides elastic recoil and resilience to a variety of connective tissues, e.g., aorta and ligaments. Elastic fibers regulate activity of TGFβs through their association with fibrillin microfibrils. Elastin also plays a role in cell adhesion, cell

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migration, and has the ability to participate in cell signaling. Mutations in the elastin gene lead to cutis laxa. Fibrillins represent the predominant core of the microfibrils in elastic as well as non-elastic extracellular matrixes, and interact closely with tropoelastin and integrins. Not only do microfibrils provide structural integrity of specific organ systems, but they also provide a scaffold for elastogenesis in elastic tissues. Fibrillin is important for the assembly of elastin into elastic fibers. Mutations in the fibrillin-1 gene are closely associated with Marfan syndrome. Fibulins are tightly connected with basement membranes, elastic fibers and other components of extracellular matrix and participate in formation of elastic fibers. Tenascins are ECM polymorphic glycoproteins found in many connective tissues in the body. Their expression is regulated by mechanical stress both during development and in adulthood. Tenascins mediate both inflammatory and fibrotic processes to enable effective tissue repair and play roles in pathogenesis of Ehlers-Danlos, heart disease, and regeneration and recovery of musculo-tendinous tissue. One of the roles of thrombospondin 1 is activation of TGFβ. Increased expression of thrombospondin and TGFβ activity was observed in fibrotic skin disorders such as keloids and scleroderma. Cartilage oligomeric matrix protein (COMP) or thrombospondin-5 is primarily present in the cartilage. High levels of COMP are present in fibrotic scars and systemic sclerosis of the skin, and in tendon, especially with physical activity, loading and post-injury. It plays a role in vascular wall remodeling and has been found in atherosclerotic plaques as well.

Keywords
Elastin • Fibrillin • Fibulins • Laminin • Tenascins

The connective tissue in general is comprised of three groups of proteins: collagens, proteoglycans, and a variety of different glycoproteins. In addition to the main weight-bearing structural proteins of connective tissue – the fibril forming collagens (discussed in the Chap. 2 by Mienaltowski and Birk) – as well as the often hydrophilic role of proteoglycan proteins (discussed in the Chap. 4 on proteoglycans by Halper, and the Chap. 13 on Animal Models by Birk), growth factors (discussed in Chap. 5 by Halper), other proteins are also important for structure and signaling within the matrix tissue of the body. Several of these proteins are currently being identified as having several important functions in the developmental phase of the tissue, where these molecules can act as mediators of signaling or structural changes in the matrix tissue. Further, many of the glycoproteins have been demonstrated to play important roles not only during normal tissue physiology, but also in response to maintaining tissue homeostasis and responding and adapting to perturbations such as mechanical loading/unloading, or tissue damage and subsequent regeneration. Further, several of them are important for pathological tissue response, e.g., in cancer, fibrosis or connective tissue anomalies. Of interest as far as the adaptation of these glycoproteins is, that several of them – including collagens and proteoglycans – can be modulated in their level of expression and synthesis by the degree of mechanical loading that the specific tissue exposed to mechanical loading senses [1]. In the following pages

some basic information about these glycoproteins is provided. However, as already mentioned above, because many of these glycoproteins are active participants in the pathogenesis of a variety of soft tissue diseases they will be discussed rather briefly in this chapter as they are also described in several chapters dealing with specific disorders of soft tissues.

3.1 Fibronectin

Fibronectin (FN) is a widely distributed multidomain glycoprotein present in most extracellular matrices (ECM). It has a molecular weight of 230–270 kD, and can, in addition to its presence in the extracellular matrix, also be detected at substantial concentrations in plasma. Fibronectin is composed of types I, II, and III repeating units (FNI, FNII and FNIII). Two intramolecular disulfide bonds are formed within type I and type II modules to stabilize the folded structure. Type III modules are formed by seven-stranded β-barrel structures that lack disulfides [2, 3]. The FN units or domains mediate self-assembly and ligand binding for collagen/gelatin, integrins, heparin, fibronectin, and other extracellular molecules [4]. The 500-kDa FN dimer is formed through a pair of anti-parallel disulfide bonds at the C terminus. FN exists in multiple isoforms generated by alternative splicing. The single FN gene transcript encodes 12 isoforms in rodents and cows, and 20 isoforms in humans. Alternative splicing occurs by exon skipping at EIIIA/EDA and EIIIB/EDB and by exon subdivision at the V region/IIICS. Fibronectin is secreted in the form of soluble inactive dimers with disulfide bonds that must be activated by interaction with α5β1 and other integrins [5, 6].

Fibronectin is widely expressed in embryos and adults, especially in regions of active morphogenesis, cell migration and inflammation. Tumor cells contain in general reduced levels of fibronectin, whereas fibronectin levels are high in tissues undergoing repair (i.e., wound healing) and/or fibrosis. In the process of matrix assembly, multivalent ECM proteins are induced to self-associate and to interact with other ECM proteins to form fibrillar networks. Matrix assembly is usually initiated by ECM glycoproteins binding to cell surface receptors, such as fibronectin dimers binding to α5β1 integrin. Receptor binding stimulates fibronectin self-association mediated by the N-terminal assembly domain and organizes the actin cytoskeleton to promote cell contractility. Fibronectin conformational changes expose additional binding sites that participate in fibril formation and in conversion of fibrils into a stabilized, insoluble form. Once assembled, the FN matrix impacts tissue organization by contributing to the assembly of other ECM proteins. Fibronectin plays an important role in fibrillogenesis in regard to initiation, progression and maturation of matrix assembly. The prominent role of fibronectin in matrix assembly lies in fibronectin ability, enabled by its multidomain structure, to bind simultaneously to cell surface receptors, e.g., integrins, and to collagen, proteoglycans and other focal adhesion molecules [7]. This property also makes it possible to mediate the assembly of several extracellular matrix protein, including type I and III collagen, thrombospondin-1 and microfibrils [4]. Fibronectin is also called a "master organizer" by some investigators [4, 8]. Perhaps more important in the context of this volume is to emphasize the role fibronectin plays in the assembly of fibrillin-1 into a structured network (see below).

3.2 Laminin

Laminins are a family of large multidomain, heterotrimeric glycoproteins with molecular weights of 500–800 kDa, located in the basement membrane. Sixteen trimeric isoforms have been described in mouse and human tissues, and these isoforms vary in their cell and tissue specificity. In general, each laminin isoform consists of three chains, α, β, and γ, and each isoform exists in five, four, and three genetically distinct forms, respectively [9–11]. Most vertebrates have five α, three γ and three to six β genes [11]. The large

range in size is due to variability in the chain size: the α chains are the largest (M_r ~200–400 kDa), both the β and γ chains range in size from 120 to 200 kDa. In addition, all forms of these three chains are highly glycosylated, some have glycosaminoglycan chains attached [9, 11]. Homologous tandem repeats of structural motifs are incorporated in all laminins, with more similarities between β and γ chains. Laminins are cross- or T-shaped molecules with two or three short arms and one long arm. The short arms consist of N-terminal parts of one of the three chains and they contain laminin-type epidermal growth factor-like (LE) repeats [11] The long arm contains portions of all three chains [9]. Common to all laminins is a coiled-coil domain with about 80 heptad sequence repeats at or close to the C-terminal end. This coiled-coil domain bears homology to segments of β and γ chains and is responsible for proper assembly of the trimer [11, 12]. Assembly of the laminin molecule is also controlled to some extent by proteolytic processing prior to laminin binding to its receptors [11].

Laminins adhere to cells primarily via binding of the G domain of the α chains to integrins, dystroglycan, or sulfated glycolipids. The N-terminal globular domains of the α1 and α2 chains as well as the globular domains VI (LN) of the α5 chains can bind to several integrin isoforms (α1β1, α2β1, α3β1, and αVβ3). This process enables cell binding on both ends of laminins containing the three α chains. The laminin γ2 chain has been reported to bind α2β1 integrin. The N-terminal globular domains of some α-chains can also bind sulfatides. This type of binding may also link the laminin molecules to the cell surface. Laminins contribute to the structure of the ECM and influence associated cells in regards to adhesion, differentiation, migration, stability of phenotype, and resistance towards apoptosis. Laminin molecules interact not only with collagen type IV, integrins and dystroglycans but also with other components of the basal membrane matrix, and thus contribute to the overall structure. They can also interact with components in the underlying interstitial stroma. The cellular effects of laminins are mediated largely via ligand binding to cell membrane receptors, and this signaling can alter transcription levels of genes and even influence chromatin remodeling of gene promoters. The insoluble network formed by laminin and type IV collagen plays a structural and functional role in the basement membrane and cells associated with it. Though at this point we do not know to what extent, if any, laminins play a role in the pathogenesis of connective and soft tissue diseases it is clear that they contribute to normal function of tendons, blood vessels and other connective soft tissues. For example, this network participates in transmission of the contractile force from the skeletal muscle to the tendons [13]. A decrease in laminin in the basement membrane covering the outermost aspect of the tendon was identified in type IV collagen deficient mice. This was accompanied by formation of spontaneous tendon adhesions [14]. That laminins are, indeed, required for proper healing of tendons and other connective tissues, such as cornea, has been shown by Molloy et al. [15] and Sato et al. [16], respectively. There is some evidence indicating increased expression of β2 chain of laminin in ascending aorta in patients with Marfan syndrome [17].

Taken together laminins are not passive adhesion proteins, but rather, they actively modulate cell behavior; influence differentiation, migration, and phenotype stability. They also inhibit apoptosis by signaling via cell membrane receptors such as integrins and dystroglycan. However, the details of laminin signaling are still largely unexplored. Laminins constitute the first ECM component appearing in the developing early embryo, and embryonic laminins have found an important use as culture matrices for stem cells. Other laminins are crucial for normal function of numerous tissues and organs, e.g., nerve, epithelium, blood vessels, and kidney. The commercial unavailability of most laminin isoforms has hampered in vitro studies. However, many isoforms have been offered recently by several companies as recombinant proteins, which may enable deeper insight into functional properties. Laminins may find numerous new applications in cell biology and cell therapy research. The vast complexity of laminin effects cannot be explained solely by simple integrin binding and signaling [11].

3.3 Fibrinogen

Fibrinogen is a large, complex, fibrous glycoprotein with three pairs of polypeptide chains: Aα, Bβ and γ [18]. The chains are linked together by 29 disulfide bonds. Fibrinogen is 45 nm in length, with globular domains at each end and in the middle connected by α-helical coiled-coil rods and has M_r 340 kDa. The E-region consisting of N-terminal ends of the six chains and the D-regions consisting of the C-terminal ends of the Bβ and γ chains and a portion of the Aα chain are separated by 3-stranded α-helical coiled-coil regions [19]. Both strongly and weakly bound calcium ions are important for maintenance of fibrinogen structure and functions. The fibrinopeptides, which are in the central region, are cleaved by thrombin to convert soluble fibrinogen to insoluble fibrin polymer, via intermolecular interactions of the "knobs" exposed by fibrinopeptide removal with "holes" always exposed at the ends of the molecules. Fibrin monomers polymerize via these specific and tightly controlled binding interactions to make half-staggered oligomers that lengthen into protofibrils. The protofibrils aggregate laterally to make fibers, which then branch to yield a three-dimensional network-the fibrin clot-essential for hemostasis. X-ray crystallographic structures of portions of fibrinogen have provided some details on how these interactions occur. Finally, a transglutaminase, Factor XIIIa, covalently binds specific glutamine residues in one fibrin molecule to lysine residues in another fibrin molecule via isopeptide bonds, stabilizing the clot against mechanical, chemical, and proteolytic insults [20]. The gene regulation of fibrinogen synthesis and its assembly into multichain complexes proceed via a series of well-defined steps. Alternate splicing of two of the chains yields common variant molecular isoforms. The mechanical properties of clots, which can be quite variable, are essential to fibrin's functions in hemostasis and wound healing [21]. The fibrinolytic system, with the zymogen plasminogen binding to fibrin together with tissue-type plasminogen activator to promote activation to the active enzyme plasmin, results in digestion of fibrin at specific lysine residues. Fibrin(ogen) also specifically binds a variety of other proteins, including fibronectin, albumin, thrombospondin, von Willebrand factor, fibulin, fibroblast growth factor-2, vascular endothelial growth factor, and interleukin-1. Though its ability to bind to a variety of compounds, particularly to various growth factors makes fibrinogen a player in cardiovascular and extracellular matrix physiology [18, 22–25], fibrinogen does not appear to play a specific role in pathogenesis of disorders discussed in this volume.

Studies of naturally occurring dysfibrinogenemias and variant molecules have increased our understanding of fibrinogen's functions. Fibrinogen binds to activated αIIbβ3 integrin on the platelet surface, forming bridges responsible for platelet aggregation in hemostasis, and also has important adhesive and inflammatory functions through specific interactions with other cells [26]. Fibrinogen-like domains originated early in evolution, and it is likely that their specific and tightly controlled intermolecular interactions are involved in other aspects of cellular function and developmental biology.

3.4 Elastin

Elastin is an insoluble polymer of the monomeric soluble precursor tropoelastin. Elastin is the main component of elastic fibers in matrix tissue, and as such it is the main contributor to the elasticity of these fibers [27, 28]. Tropoelastin is encoded by a single human gene and is secreted as an ~60 kDa unglycosylated protein by a variety of cells, including fibroblasts, endothelial and smooth muscle cells, chondrocytes and keratinocytes [28]. The splicing of the primary tropoelastin transcript is tissue-specific, and thus allows for conformational and functional adjustment for each location [29]. The primary tropoelastin sequence is an arrangement of hydrophobic domains rich in valine, proline and glycine, providing elasticity to the final product, elastin. These hydrophobic domains alternate with hydrophilic domains which contain lysine residues

whose role it is to stabilize elastin microfibrils by cross-linking [30–32]. However, before this can occur tropoelastin units are chaperoned to the extracellular surface [33] where they coacervate [34] into protein-dense spherules [35] which then undergo cross-linking and fibril assembly. Ninety per cent of the final product, i.e., of an elastic fiber, consists of a central amorphous core of elastin surrounded by a layer of microfibrils composed mostly of glycoprotein fibrillin, but also of many other proteins, among them fibulins, collagen VIII, and emilins with microfibrils as well [29, 36]. Proteoglycans, including biglycan [37] and glycosaminoglycan heparan sulfate [38] have been detected within the elastic core. Moreover, it has been shown that the presence of sulfated proteoglycans within the extracellular matrix regulates elastin assembly [39]. In addition, water plays an important role not just in the three dimensional organization of elastin molecules but also in the final degree of hydration and elasticity [38]. Elastic fibers form an interconnecting fenestrated network of lamellae in the arterial media. The lamellae are layers of elastic fibers surrounded by circumferentially oriented smooth muscle cells and collagen fibers [40].

The high content of hydrophobic amino acids makes elastin one of the most chemically resistant and durable proteins in the entire body [41]. It is distributed throughout the body in the form of tissue-specific elastic networks [28]. Elastin containing fibers provide elastic recoil in tissues where repetitive distention and relaxation is a requirement for their function, and is found typically in skin, lungs, ligaments, tendons and vascular tissues [42]. The relative content of elastin can vary from around a few percent in skin, to more than 70 % in some ligament structures in the animal kingdom. Elastic fibers are essential for proper function of at least three areas. As a major structural component elastic fibers provide elastic recoil and resilience to a variety of connective tissues, e.g., aorta and ligaments. Elastic fibers regulate activity of TGFβs through their association with fibrillin microfibrils. In addition, elastin also plays a role in cell adhesion, cell migration, survival and differentiation, and can, to some extent, act as a chemotactic agent [27, 29]. Elastin, and for that matter tropoelastin as well, is also a signaling molecule. Tropoelastin inhibits proliferation of arterial smooth muscle cells, induces the formation and organization of actin stress fibers and acts as a chemotactic agent [43].

Elastin and collagen are the dominant components of the ECM in large elastic arteries, such as aorta [40]. The two compounds play different, but complementary roles in arterial physiology: reversible extensibility during cycling loading is provided by elastin [40, 44], whereas strength and the ability to withstand high pressure is the responsibility of collagen [40, 45]. The assembly of elastic fibers proceeds only during tissue development, and cedes with maturation so older tendons contain less elastin then young tendons [46, 47]. In effect that means that with aging the stiffness of arterial wall increases due to degradation and fragmentation of elastic fibers [40, 48]. Matrix metalloproteinases (MMPs) are just some of the proteases participating in this destructive process [40, 49]. Increased levels of MMP-1 and MMP-9 have been detected in aortic aneurysms [50]. Local blockage of MMP activities in animal models either by TIMP-1 [51], inhibition of MMP-2 by calpain-1 inhibition [52], or by doxycycline, an inhibitor of MMPs [53] shows potential treatment venues. Whether they can be utilized for treatment of even prevention of complications of Marfan syndrome or related disorders remains to be seen. It is thought that production of collagen increases to compensate for the elastin deficit, however, this pushes the arterial wall towards increased stiffness [40]. Increased elastin production has been documented in some animal models of hypertension, but it is either not high enough [54] or the new elastin fibers are not assembled properly [55].

Elastin gene mutations can be divided into two groups [40]. Autosomal dominant supravalvular aortic stenosis is a representative of the first group. Besides aortic stenosis, patients develop hypertension, increased arterial stiffness leading to congestive heart failure [40]. Hypertrophy and hyperplasia of smooth muscle cells in the media of the affected arteries is due to fragmentation of

elastic lamellae and changes in ECM composition [56]. This pathology is due to loss of function mutations in the elastin (*ELN*) gene [57]. Consequently, the mutant elastin protein is nonfunctional and does not interfere with the production and assembly of normal, functional elastin in heterozygous individuals who are then less affected than homozygous people [40].

An autosomal dominant form of cutis laxa belongs to the second group which encompasses disorders resulting from missense mutation, usually near the 3′ end of the transcript [40, 58, 59]. Cutis laxa and related disorders are described in more detail in Chap. 11 by Eva Morava et al. The mutant elastin interferes with normal assembly, metabolism and function of elastic fibers [59].

Lack of elastin in the body is fatal. Elastin knockout mice (*Eln−/−*) die shortly after birth with subendothelial cells accumulation blocking blood flow and with markedly increased arterial stiffness [40, 60]. The presence of additional lamellar units in heterozygous *Eln+/−* mice indicates an attempt to compensate and to remodel in a response to increased hemodynamic stress during development [61]. Fibrillin-1 hypomorphic mice (*mgR/mgR*) serve as a model of Marfan syndrome because of aneurysm formation in the ascending aorta and elastolysis in all segments of aorta [62].

3.5 Fibrillins

Because of close association of mutated fibrillin-1 with Marfan syndrome which is being discussed in detail in Chap. 6 by Cook and Ramirez, only a brief description of fibrillins is provided in this chapter. Fibrillins are a group of large extracellular glycoproteins (~350 kDa) [29] that consists of three isoforms, fibrillin-1, -2, and -3. Fibrillin molecules contain 40–80 amino acid residues, several calcium-binding epidermal growth factor (cbEGF)-domains interspersed with several eight-cysteine-containing (TB) motifs binding TGFβ [4, 29, 63]. No other extracellular proteins contain that much cysteine as fibrillins [42]. Whereas fibrillin-2 and fibrillin-3 are mostly expressed in embryonic tissues with the exception of peripheral nerves and, to lesser degree, skin and tendon [64, 65] fibrillin-1 is a protein appearing in both embryonic and adult tissues [65–67].

Fibrillins represent the predominant core of the microfibrils in elastic as well as non-elastic extracellular matrixes, and interact closely with tropoelastin and integrins, e.g., through direct bindings. Not only do microfibrils provide structural integrity of specific organ systems, but they also provide a scaffold for elastogenesis in elastic tissues such as skin, lung, and vessels [46]. Thus, fibrillin is important for the assembly of elastin into elastic fibers. The precise arrangement of fibrillin within microfibrils is a matter of speculation; several working models have been suggested to explain the architecture of microfibrils [67]. It is known that different mutations in different regions, including the propeptide sequence encoded by the C-terminal domain, of the fibrillin-1 gene lead to impaired assembly of microfibrils in individuals with Marfan syndrome [67–69]. Robinson et al. provide an excellent, more comprehensive review of these issues, including review of self-assembly of fibrillins and cross-link formation in fibrillin assembly [67]. Besides fibrillin and elastin, the two major components, many other proteins participate in the makeup of microfibrils. As noted above fibronectin in particular plays an essential role in this process, more specifically, through binding of a C-terminal fibrillin-1 region with the fibronectin gelatin-binding region [8]. It is interesting to note that homocysteinylation of fibronectin in homocystinuria reduces fibronectin dimers to monomers, and, as a consequence, impairs assembly of fibrillin and microfibrils. Similar impairment is the result of homocysteinylation of fibrillin-1 [70].

As already mentioned above, fibrillins contain several TGFβ-binding motifs, this feature makes their structure, and, in part, their function. similar to that of latent-TGFβ-binding proteins (or LTBPs) (see more in Chap. 6), [67].

Mutations in genes for fibrillin-1 and -2 lead to several disorders in people: mutation in fibrillin-1 can result in autosomal dominant Marfan and Weill-Marchesani syndromes, mutation in fibrillin-2 leads to Beal syndrome [4, 67].

3.6 Fibulins

Fibulins are a group of seven glycoproteins that are expressed and secreted by many cell types and tissues, and that are tightly connected with basement membranes, elastic fibers and other components of extracellular matrix. The members of the fibulin family are divided into class I and II, based on their length and domain structures [71]. Class II consists of short fibulins 3, 4, 5 and 7. Fibulins 3–5 participate in elastic fiber formation and are expressed during embryonic development, especially in skeletal and cardiovascular tissues [71]. This is facilitated by Ca^{2+} [72]. Fibulin-3 is predominantly found in mesenchyme that develops into cartilage and bone, fibulin-4 is markedly expressed in heart muscle, fibulin-5 highly in vasculature, and fibulin-7 is highly expressed in teeth, placenta, hair follicles and cartilage. The molecules of short fibulins contain tandem repeats of six cbEGF domains that are connected by one amino acid in a pattern similar to the one found in fibrillin-1 [73]. Fibulin 5 contains an arginine-glycine-aspartic acid (RGD) motif which mediates binding to integrin receptors on endothelial cells and vascular smooth muscle cells [74]. This step is necessary for elastic fiber assembly [71]. Fibulin-5 also inhibits α5β1 and α4β1 fibronectin receptor-mediated downstream signaling [71]. The C-terminal fibulin module (which, by the way, is present in all fibulins) contains an elastic-binding domain in fibulin-5 [75]. The same module in fibulin-5 also interacts with lysyl oxidase-like 1, 2 and 4 (Loxl 1, Loxl 2 and Loxl4), enzymes playing crucial role in cross-linking [76, 77] whereas it is the N-terminal domain responsible for binding to Lox in fibulin-4 [78]. Lysyl oxidases, including those binding to fibulin-5 and -4 mediate cross-linking of tropoelastin monomers into insoluble elastin polymer [79]. The binding between the C-terminal module of fibulin-3 and tissue inhibitor of metalloproteinase 3 is another example of close relationship between a short fibulin and connective tissue metabolism [80]. The level of fibulin-5 is particularly high in the cardiovascular system and lung, though fibulin-4 is expressed in the outer layer of media of large blood vessels, and fibulin-3 appears in capillaries, skin and the basement membrane [71]. The participation of fibulin-5 in elastogenesis is solely due to its exclusive binding to tropoelastin but not to polymerized elastin in vitro [75]. Its role is inhibition of excessive tropoelastin coacervation into large aggregates, and consequently this allows for integration of microassembles of tropoelastin into the microfibril scaffolding [71]. In addition to fibrillins-1 and -2, as also discussed above earlier in this chapter under Elastin, fibulins are present in microfibrils of scaffolding for elastic fibers as well [81].

Fibulins serve not only as structural ECM components, but also act as mediators of several cellular processes, such as cell growth, differentiation, angiogenesis and tumor growth. Thus they serve as modulators of cellular behavior and function [82]. The molecular mechanism of fibulin activity is not fully explained, but high levels of fibulin are often observed in cartilage, especially during development. Fibulin-1 (molecular weight around 100 kDa) was originally thought to be an intracellular molecule linking cytoskeletal components to β integrins, but later it was shown that fibulin-1 was also present in fibril matrix surrounding fibroblasts in culture as well as in embryos [83]. Fibulin-2 demonstrates some overlap with fibulin-1, but its expression is more prominent in the developing heart, both aortic and coronary vessels [84]. Studies in animals lacking fibulins demonstrate importance of these glycoproteins in pathogenesis of a variety of developmental and pathological processes, e.g., impaired tissue elasticity, altered vision and reduced vascular formation.

The role of fibulins and elastin in several human diseases is being discussed in several chapters of this volume.

3.7 Tenascins

Tenascins are ECM polymorphic glycoproteins with high molecular weights between 150 and 380 kDa. They are a family of multimeric proteins labeled as tenascin-C, -R, -W, -X and -Y

[85–87]. Tenascins are composed of identical subunits built from variable numbers of repeated domains, including heptad repeats, EGF-like repeats, fibronectin type III domains and a C-terminal globular domain similar to that seen in fibrinogens. Whereas tenascin-R is predominantly found in the central nervous system, the other members of the tenascin family are found more widespread in connective and soft tissues in the body. With regards to tenascin-R, its expression is predominantly present during development of the CNS. Tenascin-X and –Y are predominantly seen in skeletal muscle connective tissue, and tenascin-C and –W have both been observed in a variety of developing tissues, and a large interest has been invested in these tenascins in relation to tumor development and growth, where they play important roles.

The first described tenascin was the C isomer. Tenascin-C is a large monomer of M_r 300 kDa, assembled into a hexamer. As other tenascins the molecule consists of a N-teminal domain, EGF-like repeats, several fibronectin type II domains and a C-teminal fibrinogen-like globular domain [87]. Tenascin-C is transiently expressed in the mesenchyme around developing organs such as kidney, teeth and mammary glands. It is present in the periostium, ligaments, tendons, myotendinous junctions, smooth muscle and perichondrium both during embryonic development and in adult tissues. However, expression of tenascin-C in the adult tissue is generally low, only to be transiently elevated upon tissue injury and often down-regulated again after tissue repair is complete. Although tenascin-C shares structural relationship to fibronectin, it differs in adhesive function. Where fibronectin is adhesive in nature, tenascin-C is only weakly adhesive – if at all – for most cells, and it does in fact limit the fibronectin-mediated cell spreading when the two proteins are combined [88]. Tenascin-C interferes with cell spreading by inhibiting binding of fibronectin to its co-receptor syndecan-4, and integrin α5β1 signaling to FAK and RhoA is also impaired whereby focal adhesions are diminished [89–92].

The expression of tenascin-C is regulated by mechanical stress both during development and in adulthood, and its expression is predominantly present in tissues experiencing high tensile stress, such as ligaments, tendons and smooth muscle [93]. Mechanical loading of muscle induces tenascin-C mRNA and protein in endomysial fibroblasts of the affected holding muscle [94]. Tenascin-C was over-expressed in hypertensive rat arterial smooth muscle [95] and in the periosteum of rat ulnae loaded in vivo, but tenascin-C expression was low in the osteotendinous interphase of immobilized rat legs [94]. Interestingly, elevated levels of tenascin-C were found in the blood of human patients with rheumatoid arthritis [96], and in synovial fluid after injury to the human and canine knee [97].

In relation to ECM tissue damage, tenascin-C has been demonstrated to play different roles that can mediate both inflammatory and fibrotic processes to enable effective tissue repair. For example, tenascin-C makes a prominent appearance in pathological heart conditions. Though barely expressed in the normal adult heart its level increases in the heart after myocardial infarction, during myocarditis, hypertensive heart disease, to name just a few examples [87]. According to the current hypothesis tenascin-C is directly involved in ventricular remodeling through releasing cardiomyocytes from the adherence to the extracellular matrix and through upregulation of matrix metalloproteinases [87, 98]. A high level of expression of tenascin-C in cardiac tissues correlates with poor patient prognosis [99]. Interestingly, tenascin-C was found in calcified aortic valve, together with matrix metalloproteinase-12 where they likely contribute to the fragmentation of elastic fibers [100].

Tenascin-X differs from tenascin-C and -R in that it is less glycosylated, and that it is present in almost all tissues, and especially widely expressed in developing fetal tissues. It is highly expressed in skeletal muscle, heart, tendon and skin. It plays an interesting role in relation to physical activity, as it is known to be upregulated in skeletal muscle in relation to acute mechanical loading and known to be present in tissues that are subjected to high stress [101, 102]. Tenascin-Y is an avian equivalent of tenascin-X [85, 103].

Overall, tenascin proteins are found to be dys-regulated in many pathological conditions like cancer, heart- and vessel disease, as well as in connective tissue diseases with manifestations in skin, tendon and muscle like e.g., special forms of Ehlers-Danlos syndrome (more discussed in Chap. 9) and Dupuytren disease [104]. Further, tenascins have been shown to be important in regeneration and recovery of musculo-tendinous tissue, in that they possess a de-adhesive effect whereby they potentially can contribute to a coordinated tissue reorganization and build-up [105]. It has been suggested that they "orchestrate" muscle build up after injury [106]. Thus it is likely that tenascins are important for ensuring mechanical properties of weight bearing ECM as well as ensuring an optimal recovery of ECM after mechanical injury.

3.8 Thrombospondins

Thrombospondins (TSPs) are a group of five modular glycoproteins, each one of them encoded by a separate gene [107–109]. TSP-1 and TSP-2 form group A, and TSPs 3–5 are in group B. Their binding to various components of the extracellular matrix, such as heparan sulfate proteoglycans, and to numerous cell membrane receptors enables TSPs to modulate cell functions in a variety of tissues [107]. They are considered to be "adhesion-modulating" components of the extracellular matrix [110].

In particular, we will discuss TSP-1 and TSP-5 as their involvement in metabolism of the extracellular matrix is pertinent to issues discussed in this volume. The activation of latent TGFβ by TSP-1 plays an important role in wound healing, and also in pathogenesis of fibrotic processes in kidney and heart in diabetes [111, 112]. Increased expression of TSP-1 (accompanied by increased TGFβ activity) was observed in fibrotic skin disorders such as keloids [113] and scleroderma [114].

TSP-1, normally stored in platelets, is released from their α-granules upon injury so it can participate in tissue repair [115]. It is a homotrimer of three 150 kDa subunits. Each unit is composed of N-terminal laminin G-like domain, and in the last 650 amino acids, of several EGF-like domains, 13 calcium-binding repeats and a globular L-type lectin-like domain. These regions in the last 650 amino acids are usually referred to as the C-terminal or "signature" region [110]. With glycosylation the size of TSP-1 balloons to staggering M_r ~450 kDa [116]. Its expression in adult organism is minimal (except for storage pool in platelets) and is upregulated only as a result of injury [117] and/or chronic disease [116, 118]. TSP1 binds to many cell membrane receptors, including CD47 [116], integrins [119], heparan sulfate and LDL [120]. TSP-1 not only binds to latent TGFβ through thrombospondin repeats, but it also activates this growth factor [121]. It is thought that TSP-1 facilitates presentation of TGFβ to the TGFβ receptor [115]. TSP-1 was shown to upregulate type I collagen expression through its N- and C-terminal domains which may explain the sometimes opposing cellular responses stimulated by TSP-1 [115, 122]. TGFβ activity induced by TSP-1 is a normal process during early tissue repair, however, if TSP-1 expression persists in later stages of wound healing fibrosis may prevail [115]. In addition, TSP-1 regulates activity of several other growth factors, most notably, VEGF, EGF and PDGF. In particular, TSP-1 plays an important role in transactivation of EGF receptors in epithelial and endothelial cells, and thus can disrupt endothelial barrier [123]. Though TSP-1 has hypertensive effect on cardiovascular system and is known to play a role in pathogenesis of atherosclerosis and peripheral vascular disease [124], the activity is mediated through control of nitric oxide synthesis (and thus increasing arterial resistance), rather than through an impact on or binding to a structural component of the blood vessel wall [124]. TSP-2 is involved in collagen fibril assembly and is capable of inhibition of angiogenesis and protease activity, but unlike TSP-1 it does not activate TGFβ [87].

However, there is at least one syndrome where a mutation in a gene encoding an enzyme responsible for proper TSP-1 function leads to structural changes which form the basis of the so called Peters Plus syndrome. This syndrome is an

autosomal recessive disorder phenotypically characterized by eye defects, short stature, developmental delay and cleft lip due to a mutation of a gene encoding a β1,3-glucosyltransferase which adds a glucose to *O*-linked fucose (and producing a rare glucose-β 1,3-fucose disaccharide) and which is responsible for glycosylation of thrombospondin type 1 repeats [125, 126]. Beside TSP-1, properdin, F-spondin, some members of a-disintegrin-and-metalloproteinase-with-thrombospondin-like-motifsfamily(ADAMTS-13 and ADAMTSL-1) carry the same disaccharide [125, 126]. Heart defects, such as hypoplastic left heart syndrome [127], patent ductus arteriosus, and atrial septal defect are present is some variants [128]. Though the eye involvement is usually characterized by anterior eye chamber defects leading to glaucoma [125, 128], corneal pathology has been recognized in some cases as well, and then it consists of intracorneal fibrosis [129] and keratolenticular adhesions [125, 128].

3.9 Cartilage Oligomeric Matrix Protein (COMP) or Thrombospondin-5

COMP or thrombospondin-5 belongs to the family of 5 extracellular calcium- and glycosaminoglycan-binding proteins that play a role predominantly during development, angiogenesis and wound healing. It consists of five identical subunits that are linked together at their N-terminal pentamerization end to result in an almost "star-like" structure and has M_r ~524 kDa [130]. COMP shares a conserved multidomain architecture in its C-terminal region with TSP-1 [110]. It also contains eight calmodulin units, four EGF-like repeats, and a globular C-terminal domain [130, 131], and the 5 "arms" have on their C-terminal end high affinity binding sites for type I, II and IX collagen [132, 133], and for fibronectin [134]. Thrombospondin-5/COMP is present primarily in cartilage, and has been suggested to be important in relation to cartilage turnover and pathogenesis of osteoarthritis [135]. It is also expressed in other connective tissues like tendon, especially if the tissue has undergone strenuous mechanical loading [136, 137]. The exact role of COMP in the fibril formation and assembly in the extracellular matrix is becoming better understood, and it is thought that COMP facilitates the joining of collagen molecules during formation of fibril structures [137, 138]. It has been shown that high levels of COMP are present in fibrotic scars and systemic sclerosis of the skin [136, 139]. It has been suggested that a very high concentration of COMP can in fact inhibit collagen fibril formation [115].

COMP is expressed in normal tendon where its mRNA is confined to tenocytes and the protein itself was located in the normally aligned fiber structures together with type I collagen. Virtually no COMP (and no type I collagen), but only type III collagen was found in the normal endotenon [137]. Physical activity leads to increased expression of COMP, at least in the equine tendon [136], as do pathological processes. High levels of COMP were identified in the synovial fluid obtained from the sheaths of the equine superficial digital flexor tendons diagnosed with synovitis [140]. Likewise, injury to superficial digital flexor tendons leads to increased expression of COMP, and type I and III collagens in the endotenon and high levels of all three molecules can be visualized in the injured and granulation tissue [137]. Rock et al. have shown that COMP promotes attachment of ligament cells and chondrocytes to components of the extracellular matrix using two mechanisms which involve CD47 and integrins. Such data indicate an important role for COMP in formation of structural scaffolding, an essential step in cell attachment to the extracellular matrix and in matrix-cell signaling [131].

In addition, new data indicate that COMP, and its degradation by ADAMTS-7, plays an important role in vascular remodeling [141]. COMP has been found in atherosclerotic plaques and lesions forming in arteries undergoing re-stenosis [142], together with SLRPs, such as decorin [143]. It has been suggested that COMP promotes differentiation of vascular smooth muscle cells and that binding and degradation of COMP by ADAMTS-7 in injured arteries enables migration of vascular smooth muscle cells and neointima formation. The hope is that ADAMTS-7

may be suitable as a therapeutic agent in combating restenosis of atherosclerotic blood vessels after angioplasties and related procedures [141]. More recent study from the same laboratory shows that COMP inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2 and that the COMP in atherosclerotic arteries story is a little bit more complicated than initially thought [144].

Though COMP has been involved in metabolism of multiple tissues, including cartilage, tendons and blood vessels the only mutations in the COMP gene known to be responsible for pathological conditions so far identified are those affecting the skeleton, such as pseudoachondroplasia and multiple epiphyseal dysplasia [131, 145].

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Proteoglycans and Diseases of Soft Tissues

4

Jaroslava Halper

Abstract

Proteoglycans consist of a protein core to which at least one glycosaminoglycan chain is attached. They play important roles in the physiology and biomechanical function of tendons, ligaments and cardiovascular system through their involvement in regulation of assembly and maintenance of extracellular matrix, and as they participate in cell proliferation through their interactions with growth factors. They can be divided into two main groups of small and large proteoglycans. The small proteoglycans are also known as small leucine-rich proteoglycans (or SLRPs) which are encoded by 17 genes and are further subclassified into Classes I–V. Several members of Class I and II, such as decorin and biglycan from Class I, and Class II fibromodulin and lumican, are known to regulate collagen fibrillogenesis. Decorin limits the diameter of collagen fibrils during fibrillogenesis. The function of biglycan in fibrillogenesis is similar to that of decorin. Though biomechanical function of tendon is compromised in decorin-deficient mice, decorin can substitute for lack of biglycan in biglycan-deficient mice. New data also indicate an important role for biglycan in disorders of the cardiovascular system, including aortic valve stenosis and aortic dissection. Two members of the Class II of SLRPs, fibromodulin and lumican bind to the same site within the collagen molecule and can substitute for each other in fibromodulin- or lumican-deficient mice.

Aggrecan and versican are the major representatives of the large proteoglycans. Though they are mainly found in the cartilage where they provide resilience and toughness, they are also present in tensile portions of tendons and, in slightly different biochemical form in fibrocartilage. Degradation with aggrecanase is responsible for the appearance of different forms of aggrecan and versican in different parts of the tendon where

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these cleaved forms play different roles. In addition, they are important components of the ventricularis of cardiac valves. Mutations in the gene for versican or in the gene for elastin (which binds to versican) lead to severe disruptions of normal developmental of the heart at least in mice.

Keywords
Small leucine-rich proteoglycans (SLRPs) • Collagen fibrillogenesis • Aggrecan • Versican • Heart development

Proteoglycans are ubiquitous molecules consisting of a protein core to which one or more glycosaminoglycan chains are attached. Their functions are numerous: they are heavily involved in regulation of assembly and maintenance of extracellular matrix, and through their interactions with growth factors they participate in cell proliferation. They also regulate collagen fibrillogenesis and tensile strength of skin and tendons, affect tumor cell growth and invasion, influence corneal transparency, and influence neurite outgrowth among many other functions [1, 2]. In the context of this volume two functions, the heavy involvement of proteoglycans in regulation of assembly and the maintenance of extracellular matrix, and their participation in cell proliferation through their interactions with growth factors particularly in certain tissues such as tendons and arteries are perhaps the most pertinent to the topic and of most interest to us.

Proteoglycans play a more important role in the physiology and biomechanical function of tendons and ligaments than would be obvious from their low content in the tendon: they comprise less than 1 % of the dry weight of tendons and ligaments (mostly decorin and biglycan, but also aggrecan) but their presence is instrumental for proper assembly and organization of the structural backbone of tendons and ligaments [3]. Because of very low aggrecan content tendons have a lower water content (55 % of wet weight) and higher collagen content (38 %) than cartilage [3, 4]. With tendon injury the water content of the tendon increases as the composition of proteoglycans in the tendons changes, and the content of sulfated proteoglycans, including aggrecan increases [5]. Changes in proteoglycan makeup of tendon, including cartilaginous metaplasia also occur with age and loading. Calcification occurs in tendons of adult turkeys [6, 7] and also in tendons of other species that undergo high tensile forces; for example, ossified tendons were found adjacent to posterior spinal elements of dinosaurs [8]. Formation of fibrocartilage with local chondrometaplasia was found also in regions of mammalian, including human tendons wrapped around bony prominences as an effect of transverse compressive forces [9, 10].

Proteoglycans are usually divided into two large groups of small and large proteoglycans [1]. The small proteoglycans are also known as small leucine-rich proteoglycans (or SLRPs) [1, 11–13]. These compounds consist of relatively small core proteins (~40 kDa) to which one to two chondroitin or dermatan sulfate (CS or DS) or several keratan sulfate (KS) chains are attached [1, 14]. The core proteins contains numerous leucine-rich repeats (LRRs), which are 20–30 amino acid long with leucine situated in conserved positions [1].

SLRPs are encoded by 17 genes, and can be further divided into 5 classes. The members of the first two classes are involved in collagen fibrillogenesis and related processes (and because of that only those SLRPs will be discussed in this review). Decorin and biglycan, members of Class I, bind to the same site on collagen I. Class I SLRPs contain 10 leucine rich regions, and have chondroitin and/or dermatan sulfate attached to the protein core [11]. Fibromodulin and lumican, members of Class II, bind to a different site on type I collagen molecule [15–18]. The members of class II have 10 leucine rich regions in their central domain, and carry keratan sulfate chains

attached to the leucine rich regions. All of these SLRPs are present in tendon where they participate in fibrillogenesis and thus contribute to the development of proper biomechanical function [19]. The composition of amino acid sequences intervening between four cysteine residues comprising N-terminal cysteine clusters determines which class the particular SLRP belongs to [20]. In addition, proteoglycans in Classes I–III and extracellular matrix protein 2 (ECM2) contain a so called ear repeat, an LRR elongated by several residues [21]. McEwan et al. propose that the ear-repeat C-terminal motif is the hallmark of members of the true SLRP family [21].

Based on their protein homology Class II members can be further subdivided into three smaller groups. Class II SLRPs contain N-terminus clusters of tyrosine sulfate residues, and are, therefore, polyanionic. They are fibromodulin, lumican, PRELP, keratocan and osteocadherin and they bind keratan sulfate and polyalactosamine (a nonsulfated form of keratan sulfate). Their exonic organization is similar among them with three exons [12]. All of them except for PRELP contain an N-terminal extension with at least one sulfate tyrosine residue [22]. The N-terminal region of PRELP binds to heparin instead [23].

Three compounds are included in Class III. Epiphycan, opticin and osteoglycin share seven LRRs and seven exons [20]. They also possess a consensus sequence for glycanation. They usually appear in tissues as glycoproteins rather than as proteoglycans [20].

Chondroadherin, nyctalopin and tsukushi form a new non-canonical Class IV, which should not be considered true SLRPs because of a different C-terminal capping [21]. They have 11 homologous LRRs flanked by cysteine rich N-terminus. Like members of Class I, tsukushi is an inhibitor of several bone morphogenetic proteins (BMP-2, -4 and -7) [20, 24].

Another non-canonical group, Class V, includes podocan and podocan-like protein. Both are characterized by 20 LRRSs with homology to members of Class I and II. The ability of podocan to bind type I collagen makes this molecule even more similar to other SLRPs [20, 25].

Schaefer and Iozzo suggested that many SLRPs, particularly those in Classes I – III, arose by duplication of chromosomal segments. This would explain the similarity and redundancy in their function [20].

The development of knockout mice had brought increased understanding of the role SLRPs play in normal physiology and of the role of mutated or deficient SLRPs in disease. Because many SLRPs, particularly those belonging to Classes I and III, bind to several type of collagen they play important roles in the assembly of collagen fibrils, extracellular matrix function, and by extension in physiology of most, if not all, tissues and organs.

As pointed out by Schaefer and Iozzo, SLRP-linked genetic diseases lead to eye abnormalities. Interestingly, though point mutations in the human decorin gene result in congenital stromal corneal dystrophy, an autosomal dominant disorder limited to the cornea [26, 27], decorin-deficient mice exhibit abnormal collagen fibril structure in the tendon and skin, but not in the cornea [28]. The fact that the decorin in congenital stromal corneal dystrophy was found to be truncated and prone to form high molecular weight complexes, at least in vitro, and opacities in vivo [27], and thus was present, albeit defective whereas the absence of decorin mRNA or a protein itself in decorin-deficient mice [28] likely accounts for the difference in phenotype due to complete decorin deficiency.

An N-terminus with a typical cluster of cysteine residues forming two sulfide bonds is considered a marker of Class I with decorin, biglycan and aspirin as members. The Class I members have a similar gene organization with eight exon and highly conserved intron/exon junctions. ECM2, though much larger protein, has 33 % of its LRR identical to the corresponding domains of decorin, and its gene is actually linked to the gene for asporin on chromosome 9 [20].

Decorin is the main proteoglycan in tendon. It consists of three domains [29]. A single dermatan/chondroitin sulfate chain binds to an N-terminal region. A central region contains 10 leucine rich repeats forming repeating parallel beta sheets which contain a binding site for several collagen

types (type I, II, III, and VI). It contains binding sites for several other proteins such as for TGFβ [30] and EGF and its receptor [31]. In addition, the attached glycosaminoglycan chain also has binding sites for TGFβ and TNFα [32, 33]. A C-terminal region contains, just like the N-terminus, several cysteine residues [29]. Decorin is detected in tendons during the first stage of collagen fibrillogenesis [34], and remains expressed in tendons (and other connective tissues) throughout the life of an individual. Decorin regulates the size of collagen fibrils during fibrillogenesis with the purpose to limit the diameter of fibrils and modify the rate of the process [22]. Decorin knockout mice have collagen fibrils varying in dimension and thicker diameter and widely uneven shape, especially in skin and tendon. As a consequence their skin (and tendon) was fragile with reduced tensile strength [28]. As decorin expression increases with age the tendon worsens biomechanically because of changes in fibril structure and diameter. Paradoxically, tendons from decorin-null mice show less decline in their biomechanical parameters with aging than tendons from intact mice [35].

Decorin has many other functions besides its regulation of fibrillogenesis. It inhibits TGFβ activity, presumably by sequestering the growth factor [32]. It participates in regulation of cell proliferation and immune response [1]. Decorin can substitute for absent biglycan, at least in animal models [36].

Two chondroitin or dermatan sulfate chains are attached to the protein core of biglycan [37]. As a proper member of Class I SLRP its protein core consists of 10 leucine-rich repeats [11, 38]. Its central domain contains binding sites for type I and VI collagens [16, 39]. The affinity of biglycan binding to collagen is similar to decorin and is independent of *N*-linked oligosaccharides present on the biglycan molecule [16]. Similarly to decorin it interacts with TGFβ and TNFα through binding sites on the core protein and the dermatan sulfate chain attached to the core protein [32, 33]. Biglycan is present at particularly high levels in developing tendon. At E16-18 of mouse embryonic development tendons are already quite prominent and express high levels of biglycan where it is coexpressed with collagen VI, and though the level of biglycan then declines it remains detectable postnatally [40]. Together with aggrecan (see below) biglycan is present in higher concentrations in the fibrocartilaginous region of tendon [41]. Several studies have shown that abnormal collagen fibrils and their assembly in bone, dermis and tendon are also accompanied by impaired function in these systems in biglycan- deficient mice [36, 42–44]. The stimulation of BMP-4 expression and inhibition of BMP-2 expression by biglycan leads to proper tendon development in the embryo [12]. In biglycan (and fibromodulin) deficient mice BMP-2 which is released from biglycan-controlled inhibition (at least in tendon) drives inhibition of tendon development [20, 45]. Both decorin and biglycan are also expressed in healthy arteries [46, 47]. The adventitia of aorta is a major site for biglycan deposition [38]. However, biglycan plays a somewhat sinister role in the development of atherosclerotic plaques and aortic valve stenosis. Biglycan promotes lipid deposition through binding to Toll-like receptor, induction of cytokine production and inflammation. It binds to Toll-like receptors 2 and 4 of macrophages, and thus functions as one of mediators of innate immunity [20]. It also induces the expression of BMP-2 and alkaline phosphatase in human aortic valve interstitial cells primarily through Toll-like receptor 2, and thus contributes to the osteogenic process and calcifications during pathogenesis of aortic valve stenosis [48]. A diminished LDL binding ability of biglycan (and versican, another LDL-binding proteoglycan) and the subsequent release LDL from human aortic lesions can be induced by ADAMTS-5 activity [49]. Though primary deficiency of biglycan or mutation in the biglycan gene has not been reported in people, the risk for aortic dissection may be X-linked [50], and aortic dissection and rupture are, indeed, more frequent in women with Turner syndrome [51]. In contrast, it is the biglycan-deficient male mice rather than the females which suffer aortic rupture due to abnormal collagen fibrils with great variation in size and shape [38].

Asporin, another member of Class I, competes with decorin for collagen binding but because of

its ability to bind calcium [52] it plays a role in pathogenesis of osteoarthritis and other joint and bone disease rather than in tendon disorders [53]. Interestingly, it has no glycosaminoglycan chain attached to its protein core. It is a negative regulator of TGFβ and BMP-2 activities [53].

All known members of Class II bind to fibrillar collagen through the leucine-rich region, and both fibromodulin and lumican bind to the same site within the collagen molecule [22]. Fibromodulin is associated with types I and II collagen fibrils, as it binds to the gap regions of both collagens, at sites different from collagen binding sites for decorin and biglycan [54, 55]. It has multiple binding sites for type I collagen, but only one binding site for type II collagen [18]. It impedes fibril formation of these two collagens [56]. The keratan sulfate chains bound to fibromodulin protein core confer negative charge onto this proteoglycan. In addition, the presence of sulfated tyrosine residues together with numerous acidic amino acids at the N-terminus endows this region with properties similar to negatively charged heparin [57]. As a consequence, this domain of fibromodulin binds growth factors and cytokines, such as FGF2, IL-10 and oncostatin M, and likely plays a role in interactions with collagen [22].

Lumican, an SLRP closely related to fibromodulin, though present in tendon, plays a major role in connective tissues of cornea, skin and muscle where by virtue of multiple keratan sulfate chains attached to the core protein it retains water, and thus provides hydration and resilience to these tissues. Lumican-deficient mice have corneal clouding and skin laxity, but only minimal tendon impairment [58]. Like fibrobromodulin lumican is involved in fibrillogenesis of types I and II collagen [18] and substitutes for fibromodulin in fibromodulin-deficient mice [59]. Similar to biglycan, lumican binds to Toll-like receptor as part of the antigen recognition process [20].

Studies on mice deficient in one or two the genes for the four SLRPs (i.e., decorin, biglycan, fibromodulin and lumican) showed somewhat overlapping role for these proteoglycans in fibrillogenesis. Deficiency in at least one of them has an effect on morphology and diameter of collagen fibrils. For example, the most dramatic effect of decorin deficiency is the emergence of irregular fibrils with large diameter and a decrease in skin and tendon tensile strength [28]. This is due to uncontrolled lateral fusion of thin and thick fibrils [29]. Abnormal fibril formation is also seen in biglycan-deficient mice [44], and such abnormalities are accentuated in double knockout mice for both decorin and biglycan [42]. Such findings confirm that these proteoglycans play a major role in at least the third stage of fibrillogenesis where they limit lateral fibril growth. Biglycan deficient mice have normal skin but show reduced bone density [42]. Lumican is able to substitute functionally for fibromodulin in fibromodulin-deficient mice. In contrast, large diameter collagen fibrils forming disorganized matrix in cornea and skin were found in lumican-deficient mice. It is interesting to note that this did not lead to loss in tendon biomechanical function and that fibromodulin did not substitute for loss of lumican function [60].

Keratocan, a less described member of Class II, has been identified in the tendon in the fibrocartilage, in the tensional region and in the endotenon [41]. It is interesting that tendon keratocan is poorly sulfated in the tendon and highly glycosylated in the cornea, perhaps reflecting differences in its function in those two tissues [41].

Aggrecan and versican, two representatives of the group of large proteoglycans binding hyaluronan, are the main proteoglycans synthesized in cartilage where they provide resilience and toughness. Aggrecan binding to hyaluronan is mediated by a link protein binding to the globular G1 domain of aggrecan. The second domain or G2 though quite homologous to G1 does not have the ability bind hyaluronan, and its function is not understood. G3 is located at the C-terminal end of the molecule and has homology to C-type lectin [22]. This domain binds and interacts with extracellular matrix proteins with EGF-repeats in their molecules, such as fibrillins, fibulins and tenascins [61]. A point mutation in this lectin region leads to chondritis dissecans due to loss of interactions with fibulin-1, fibulin-2, and tenascin-R, and it is characterized by fragmentation

of articular cartilage and subsequent dislocation of subchondral bone from the joint surface early in life [62]. Aggrecan is a highly glycosylated molecule with numerous CS and KS chains attached to its large core protein (M_r ~220 kDa). In addition, aggrecan contains a variable number of *O*- and *N*-linked oligosaccharides [14]. Up to 100, somewhat heterogenous, CS chains are attached to the CS1 and CS2 domains and even undergo further increase in diversity in diseases such as rheumatoid arthritis [22]. Because the numerous negatively charged keratan sulfate chains attached to aggrecan protein core constitute the collagen binding region of aggrecan, the ECM acquires high osmotic pressure. The tissue then attracts water. The resulting high tissue hydration renders the tissue resistant to compressive loading [63]. This resilience is important not only in cartilage but also in regions of weight bearing tendons experiencing compression [4], and in the walls of atherosclerotic blood vessels experiencing high shear [64].

Several aggrecanases cleaving specific sites on the protein core participate in degradation of aggrecan. They are members of the ADAMTS family of proteinases and are primarily active in the cartilage [65]. The activity of ADAMTS was also demonstrated in tendons where some of them, particularly ADAMTS-4 and -5 cleave aggrecan at specific sites, especially in tendons with abnormally increased content of aggrecan [66–68].

New acquisition of aggrecan also happens with age [41], and it is a sign of tendons or their regions undergoing remodeling into fibrocartilage [4]. In addition, a different form of aggrecan has been found in tensile regions of tendon: it lacks the G1 domain due to proteolytic degradation by aggrecanases [4, 41, 69]. Interestingly, comparative explant cultures of tendons and of articular cartilage have shown much higher turnover of aggrecan in tendons than in cartilage. This is associated with release of aggrecan G1-containing metabolites and high levels of hyaluronan from tendon matrix [41]. This is likely due to differences in composition between tendon and cartilage aggrecans. It has been suggested that tendon aggrecan is more susceptible to aggrecanase degradation due to the higher content of non-sulfated chondroitin sulfate disaccharide isomers and less keratan sulfate [41].

Versican is another member of the hyaluronan-binding PGs [14, 70]. It is expressed in many tissues, especially in fast growing cells of soft tissues. Versican is found in the dermis of skin and in the media of the aorta, and in lower levels in tendon. It appears transiently during mesenchymal condensation in developing chicken limb buds [71]. An increase in versican content leads to expansion of ECM and to increased viscoelasticity of pericellular matrix that supports cell-shape changes necessary for cell proliferation and migration. ADAMTS-1 and -4 were shown to cleave also versican, in addition to aggrecan [72]. Similarly to aggrecan, versican is present in tensile regions of tendons in a catabolized form, presumably the result of aggrecanase activity. Like aggrecan, versican in this location lacks the G1 domain [69]. The presence of G3 domain which contains a C-type lectin sequence and binds multiple components of the extracellular matrix including fibulins 1 and 2 [73, 74], and tenascin C [61] indicates that versican is involved in structural organization of the tendon [41]. Versican also interacts with CD44, integrin and EGF receptors through another binding site [75]. It also stabilizes the presence of TGFβ in the extracellular matrix and regulates TGFβ signaling [76].

Though neither aggrecan nor versican have been implicated as direct culprits in the pathogenesis of systemic diseases affecting soft connective tissues, including those involving cardiac valves and aorta, such as Marfan or Ehlers-Danlos syndrome, these two proteoglycans do play important roles in the structure and physiology of the cardiovascular system. Thus far we know that mutations in fibrillin I lead to Marfan syndrome and mitral valve prolapse (for more details see Chaps. 6 and 8 by Cook and Ramirez, and by Wheeler et al., respectively). Mutations in type III collagen and tenascin X may contribute to mitral valve prolapse and pulmonary valve stenosis [77]. Because these molecules are tightly associated with aggrecan and versican it is likely that the products of the mutated genes affect the function of these proteoglycans as well.

The extracellular matrix in cardiac valves is organized into three overlapping layers (fibrosa, spongiosa and ventricularis). Collagen fibers form the fibrosa, or arterial layer of the valvular cusp. The centrum called spongiosa is composed of loosely deposited proteoglycans, including both aggrecan and versican. Finally, the ventricularis, facing the ventricle, has a high content of elastin [77, 78]. The precise composition of these layers changes during embryonic remodeling. The expression of aggrecan increases during cushion elongation and this proteoglycan is also present in the fibrosa and spongiosa during the cusp remodeling. This is associated with differential expression of other components of cardiac valves such as type III collagen, tenascin and elastin as already referred to above [77]. The expression of aggrecan and versican is controlled by at least two transcription factors, Twist1 [79] and TBx20 [80]. Recent studies show that versican plays an important role in cardiac development. Several enzymes, such as matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) and ADAMTS-5 and ADAMTS-9 process and cleave versican which is particularly relevant in the development of certain experimental valve defects (see below) [81, 82]. A lethal mutation in the versican gene was described in mice which die in utero due to hypoplastic endocardial cushions and malformations of the right cardiac chambers [83]. However, some mutant mouse embryos with a mixed background and lacking only the A subdomain of the G1 domain survived to the neonatal stage with a ventricular septal defect [84]. Versican in these embryos had a decreased ability to bind hyaluronan. The versican splicing forms were decreased in the atrioventricular canal cushion and the ventricular septa. The normal endocardial mesenchymal transition from highly proliferative endocardial cells to less proliferative remodeling mesenchymal cells occurring as part of normal cardiac development [77] was disrupted as endocardial cells persisted in their proliferative state due to the lack of the versican A subdomain [84].

However, the defect or mutation does not have to necessarily reside in versican or its gene for versican to disrupt normal heart development. Versican interacts closely with elastin, the major component of the ventricularis layer of cardiac valves. Versican limits elastogenesis as it interferes with elastic fiber assembly [75, 85]. There was excessive proteoglycan accumulation in the annulus region of the developing aortic valve of elastin-insufficient mice (heterogenous for elastin deficiency, designated as *Eln*+/−) due to increase in intact and cleaved versican accompanied by an increase in MMP-2 and MMP-9. This was in sharp contrast to wild type mice where the proteoglycan in the annulus was identified as aggrecan and no versican, intact or cleaved, was detected [81]. As Krishnamurthy et al. point out versican and aggrecan complement each other and so may substitute for each other's function if necessary [81].

Whether this would be the case in other cardiac valves and whether disorders of other components of the extracellular matrix expressed in Marfan, Ehlers-Danlos and other, related syndromes lead to imbalance between aggrecan and versican expression and function needs to be ascertained in the future.

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Advances in the Use of Growth Factors for Treatment of Disorders of Soft Tissues

5

Jaroslava Halper

Abstract

Repair and healing of injured and diseased tendons have been traditionally fraught with apprehension and difficulties, and often lead to rather unsatisfactory results. The burgeoning research field of growth factors has opened new venues for treatment of tendon disorders and injuries, and possibly for treatment of disorders of the aorta and major arteries as well. Several chapters in this volume elucidate the role of transforming growth factor β in pathogenesis of several heritable disorders affecting soft tissues, such as aorta, cardiac valves, and tendons and ligaments. Several members of the bone morphogenetic group either have been approved by the FDA for treatment of non-healing fractures or have been undergoing intensive clinical and experimental testing for use in healing of bone fractures and tendon injuries. Because FGFs are involved in embryonic development of tendons and muscles among other tissues and organs the hope is that their testing would lead to the development of some new treatment strategies providing that we can control angiogenicity of these growth factors. The problem, or rather question regarding practical use of IGF-I in tendon repair is whether IGF-I acts independently or under the guidance of growth hormone. FGF2 or PDGF alone or in combination with IGF-I stimulated regeneration of periodontal ligament, a matter of importance in Marfan patients with periodontitis. In contrast, VEGF appears to have rather deleterious effect on experimental tendon healing, perhaps because of its angiogenic activity and stimulation of matrix metalloproteinases, proteases whose increased expression has been documented in a variety of ruptured tendons. Other modalities, such as local administration of platelet-rich plasma (PRP) and/or of mesenchymal stem cells have been explored extensively in tendon healing. Though

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treatment with PRP and mesenchymal stem cells has met with some success in horses (who experience a lot of tendon injuries and other tendon problems), the use of PRP and mesenchymal stem cells in people has been more problematic and requires more studies before PRP and mesenchymal stem cells can become reliable tools in management of soft tissue injuries and disorders.

Keywords

Tendon repair • Transforming growth factor β (TGFβ) family • Bone morphogenetic proteins (BMPs) • Fibroblast growth factors (FGFs) • Insulin-like growth factor I (FGF-I) • Platelet-derived growth factor (PDGF) • Vascular endothelial growth factor (VEGF) • Platelet-rich plasma (PRP) • Stem cells

5.1 Basics of Tendon Repair

One of the challenges facing patients with tendon injuries either due to trauma or joint laxity in Ehlers-Danlos syndrome is the lack of good options for management and treatment of such disorders. Repair in the tendon generally follows along the classical pathways for wound healing marked by a sequence of inflammation, proliferation and remodeling. As a result of remodeling, type I and type III collagen are laid down first in the form of small diameter fibrils [1]. Tensile strength increases when crosslinks are re-established at 3 weeks after the injury [2], though the healed tendon does not regain the original organization or biomechanical function even years later in most cases [3]. Surgical repair is usually incomplete as well, resulting in decreased stiffness and adhesion formation [4, 5]. Patients with Marfan syndrome also suffer from joint problems and from periondontitis, or inflammation of the gingiva, periodontal ligament and alveolar bone loss which does not accompany dental caries [6].

Though less obvious than in other tissues, healing of tendons and ligaments occurs through two pathways. In the extrinsic pathway cells participating in tendon repair are recruited from outside the injury site. In this case the process ends with a scar, and, in many cases, with adhesions hindering the normal gliding motion of tendon in the sheath [7, 8]. The intrinsic pathway leads, at least in theory, to regeneration of the tendon without scarring and adhesion formation. It consists mostly of proper organization of collagen deposited into the wound bed during its fibroblastic/proliferative and remodeling stages [7]. During the initial or inflammatory phase, lasting about 2 weeks, neutrophils and fibroblasts migrate into the wounds. Accumulation of hyaluronan makes tendon repair distinct from repair in other tissues [9]. It is the first macromolecule appearing in the wound bed. It promotes cell differentiation and growth [10], and contributes to scarless healing [7]. The proliferative or fibroblastic stage lasts for several weeks and is marked by proliferation and differentiation of epitenon cells until they form a multicellular layer enwrapping collagen bundles at severed or injured ends of the tendon. Those fibroblasts then synthesize and secrete growth factors, such as TGFβ and IGF-I which in turn stimulate the production of collagen fibrils which then undergo proper assembly [7, 11, 12]. At this time type III collagen appears in addition to type I collagen and participates in fibrillogenesis [7]. Inherently the emphasis on preservation of biomechanical function of intact and healing tendon results in its poor vascularization [13, 14].

In addition, a distinction has to be made between acute and chronic injuries of both tendons and ligaments as the pathogenesis and pathology differ [15]. Whereas tearing of collagen fibers, hemorrhage and inflammation

with progression through standard stages of tissue repair are typical for the appearance and subsequent healing of acute injuries of tendons and ligaments [15, 16], chronic injuries of tendons and ligaments are less well defined [15]. Their incidence increases with age and they are associated with overuse. Their hallmarks are degenerative change such as collagen disruption, mucoid or proteoglycan accumulation, neovascularization. Inflammation is conspicuously absent [17]. Chronic ligament injuries are thought to arise from chronic instability secondary to failed healing of acute injuries, often involve fiber stretching or tearing and joint laxity, though inflammatory changes are sometimes present [18, 19].

As a consequence, even a seemingly well healed tendon does not return to its original structural integrity and full function [20, 21]. Beside traditional measures such as surgery, exercise or immobility, administration of painkillers, advances in basic research on growth factors markedly expanded our knowledge of tissue repair, and this started bringing solutions to practical problems. Growth factors are numerous, and it seems that each year we add more of them to our already extensive catalog of small, mostly glycosylated proteins involved not only in cellular proliferation, but also in embryonic development and in maintaining or modulating differentiated functions of tissues and organs throughout the entire lifespan [22]. Most growth factors are ubiquitous and pleiotropic, and a comprehensive review of their activities to discuss would go beyond the scope of this volume. Growth factors, such as transforming growth β1 (TGFβ1), bone morphogenetic proteins (BMP), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs) and insulin-like growth factor I (IGF-I) are produced by tenocytes, inflammatory white blood cells, and are released from platelets during wound-induced degranulation [22]. These growth factors (and some other as well) actively participate in proliferation and formation of extracellular matrix, and of connective tissues, including tendons and ligaments [23].

Because of their sheer number and complex activity and effects, only a few whose role in tendon healing and whose function in soft tissue disorders has been understood the best will be described here.

5.2 Transforming Growth Factor β (TGFβ) Family

This numerous and complex group of growth factors is indispensible during embryonic development and beyond, and is usually divided into four groups [22]:

1. Mullerian inhibitory substance group regulates Mullerian duct regression in male embryos.
2. inhibin/activin group includes inhibin (which blocks follicle stimulating hormone (FSH) release by pituitary cells) and activin (which stimulates FSH release by pituitary cells).
3. Vg-related proteins regulate primarily embryonic development and cell differentiation. This group includes bone morphogenetic proteins or BMPs (which regulate bone and tendon development), dorsalin (which regulates neural tube differentiation); growth differentiation factors or GDFs; DPP or decapentaplegic transcript (regulates dorsal-ventral patterning in Drosophila); and VgI gene (inducer of mesoderm development from ectoderm in Xenopus).
4. TGFβ family contains three isoforms, TGFβ1-3.

All members of the TGFβ superfamily transduce their signals through serine/threonine kinase type I and type II receptors, designated as TβR-I and TβR-II, or their equivalent cell membrane compounds [24]. Initial binding of TGFβ to TβR-II leads to recruitment of TβR-I and formation of a heterotetramer. This configuration triggers a phosphorylation cascade starting with the TβR-II mediated activation of the TβR-I kinase, and of the several Smad proteins and other molecules [25]. As an alternative to the division into the four groups outlined above the members of the TGFβ family can be divided into two large clusters depending on the type of TβR-I they bind to and the Smad proteins they activate [25].

According to this classification, TGFβs 1-3, activins, GDFs 8, 9 and 11, BMP3 and *nodal* form the first class which, through TβR-II binding, turns on one type of TβR-I receptor which then activates Smad 2 and 3. The second class includes presumably more distantly related proteins, which include BMPs 2, 4-10, and GDFs 1, 3, 5, 6 and 7 [25]. These growth factors bind to two types of serine/threonine kinase receptors which have diversified somewhat from the original TβR-I and TβR-II scheme. There are as many as three type I receptors (BMPR-1a, BMPR-1b and ActR-1), and three type II receptors (ActR-II, ActR-III and BMPR-II). The type I receptors to which the BMP/GDF ligands bind activate Smads 1, 5, and 8 [25, 26]. In addition, these ligands are known to bind to multiple type I and type II receptors [25]. According to another classification BMPR-1a receptor is also known as activin receptor-like kinase 3 (ALK3) and BMPR-1b is identical to ALK6 [27]. More complete review of TGFβ signaling is provided by Wheeler et al. in Chap. 8.

Only BMPs and TGFβ isoforms are pertinent to the topic of disorders of soft tissues as discussed in this volume. TGFβ1-3 isoforms share 75 % amino acid homology and are secreted as homodimers in their latent precursor form. The latency-associated peptide prevents TGFβ from binding to its receptor, and proteolytic cleavage is necessary for TGFβ activation. Active TGFβ isoforms are homodimers of $M_r \sim 25$ kDa with several disulfide bonds forming a cystic knot [28, 29].

TGFβ1 is expressed in most cells, but primarily in endothelial, hematopoietic and connective tissue cells. TGFβ1 stimulates the proliferation of mesenchymal cells (though, on occasion it inhibits their proliferation), and is a potent inhibitor of epithelial and endothelial cell proliferation, and a potent immunosuppressant. TGFβ1 is also a strong fibrogenic agent: it induces the production of collagen and other components of extracellular matrix, downregulates the expression of matrix metalloproteinases (which degrade extracellular matrix and connective tissues), and upregulates the synthesis of protease inhibitors [22].

TGFβ1 has been shown to play crucial roles in pathogenesis and pathophysiology of several syndromes and diseases as described in Chaps. 6 and 8 by Cook and Ramirez, and by Wheeler et al. in this volume on Marfan syndrome, valvulopathies and others. Though its involvement in Marfan syndrome is secondary due to its dysregulation by mutated fibrilin, its role as a mediator of substantial pathology in Marfan syndrome, makes this disorder an excellent model for studies on the role of TGFβ1 (and to lesser extent of BMPs) in proper function of cardiovascular system, and other soft tissues as well. I am not going into more details regarding TGFβ1 as the excellent chapters by Cook and Ramirez (Chap. 6 on Clinical, Diagnostic, and Therapeutic Aspects of the Marfan Syndrome) and by Wheeler et al. (Chap. 8 on Connective Tissue Disorders and Cardiovascular Complications: The Indomitable Role of Transforming Growth Factor-Beta Signaling) in this volume provide quite comprehensive overview of TGFβ activity and participation in pathogenesis of Marfan syndrome and related, and less related disorders. Though the authors concentrate on the cardiovascular system, TGFβ1 exerts similar effects on other soft tissue structures, such as bones and ligament as the two systems (cardiovascular and musculoskeletal) share certain developmental and regulatory mechanisms, and very similar extracellular matrix structure and composition [30]. However, it has been shown that TGFβ1 null mice suffer primarily from overwhelming inflammatory processes and from vasculogenesis defects rather than from musculoskeletal problems, and that they die early in life [31, 32]. BMP2 null mice fare even worse as they die in utero because of severe cardiac defects [32, 33]. Smad 1, 2, 4 and 5 deficient mice meet the same fate in utero because of cardiac defects [32, 34]. As those reports were all focused on cardiovascular systems, no description of musculoskeletal system in these mice was provided.

Because TGFβ is known to be a very powerful promoter not only of collagen synthesis, but of other components of ECM as well it is not surprising that it plays an important role in tissue repair. It appears in early stages of tendon repair during the first week post-injury [35].

Exogenous TGFβ1 has been shown to promote tendon and ligament development either alone or in combination with other growth factors, such as VEGF or BMP12 [36, 37], and though so far it has not been used in clinical practice, it does have a promise as a treatment agent in tendon and ligament injuries, at least in some studies. The addition of TGFβ- neutralizing antibody to cultures of both acellular tendon matrix, and of acellular tendon matrix co-cultured with tendon-derived fibroblast media had negative effect on biomechanical parameters of the tissue [38]. Implantation of rat muscle tissue transduced first with recombinant adenovirus encoding for TGFβ1 into rat with transected tendons of Achilles led to accelerated healing [39]. However, other studies present a somewhat different picture [40]. The overexpression of TGFβ1 and TGFβ2 in post-injury rabbit flexor tendons correlated with fibrosis and scarring which could be controlled with anti-TGFβ antibodies [41, 42].

Our understanding of TGFβ dysfunctional activity in pathogenesis of Marfan and related syndromes might lead to development of treatment modalities which would focus on blocking TGFβ1 action rather than promoting it. This could be achieved using an approach employed in chemotherapy of cancers with biologics, e.g., with monoclonal antibodies to the growth factor itself (see also in the Chap. 6 on Clinical, Diagnostic, and Therapeutic Aspects of the Marfan Syndrome by Cook and Ramirez), or with an agent blocking a step in the TGFβ1/Smad signaling pathway.

BMPs were first discovered as inducers of bone and cartilage formation [43]. Today we know that this rather large group with more than 20 members is involved in organ patterning and in formation of tissue architecture and structure during embryonic development as well [44, 45]. The group of growth and differentiation factors (GDFs) is closely related to BMPs and regulates cartilage and skeletal development [46]. Some of the GDFs turned out to be identical to certain BMPs (see below). The so called heterodimeric BMPs are composites of monomers derived from different BMP isoforms and they are primarily involved in osteogenesis [44, 47]. Both natural and recombinant BMPs have found use in orthopedics and dental surgery as they are highly osteogenic [44]. Several laboratories have shown that three BMPs (BMP14, BMP15 and BMP16), first described as growth and differentiation factors in mice (GDFs) 5-7, were able to induce tendon- or ligament-like tissue in vivo [48, 49]. GDF5 is also known as BMP14, GDF6 as BMP13, and GDF7 as BMP12 [50]. In another study, ectopic administration of the same BMPs led to formation of tendon-like structures and expression of a tenocytic marker gene [51]. GDF5 (BMP14) stimulated healing of Achilles tendon in rats [52–54]. GDF5 also induces tenogenic differentiation in human mesenchymal stem cells derived from bone marrow as judged from upregulation of genes encoding for tendon markers such as scleraxis, tenascin and type I collagen [55]. The application of BMP14 also supported periodontal regeneration through stimulation of both bone and ligament formation [56]. BMP13 in particular appears to be a potent promoter of ectopic tendon tissue formation (though with some focal ossification foci occurring in the newly formed tendon tissue) upon intramuscular injection of BMP13 [57]. BMP12 is another tendon-differentiating agent which has been used for in vivo [58] and in vitro [59, 60] tendon healing and development either by gene transfer [58, 59] or by in vitro exposure to BMP12 [60]. Apparently, whether the final product of BMP activity is tendon or bone (or cartilage) depends not only on the BMP isoform used but also on the type of mechanical stimulation. Tension or shear applied to undifferentiated mesenchymal tissues leads to the development of fibrous (tendinous) tissues, whereas cartilage appears with the application of hydrostatic compression [61].

BMP2 is a powerful osteogenic agent which has been approved by the FDA for treatment of acute open tibial shaft fractures, and has been used in the off-label treatment of non-union and acute fractures with considerable success [62], though it has some tenogenic qualities as well. Mesenchymal stem cells overexpressing BMP2 and Smad8 (a mediator of signaling of TGFβ

superfamily) have been found to differentiate into tendon cells upon expressing scleraxis, a factor stimulating tendon formation in the embryo [63]. In the same study, those cells had also the capacity to induce Achilles tendon repair in rats [63]. However, in vivo experiments have shown that BMP2 stimulated healing at the tendon-bone interface due to accelerated new bone formation rather than due to tendon formation [10, 64]. And, indeed, in yet another study BMP2 had rather detrimental effect on tendon-to-bone healing in a canine flexor tendon repair model, likely due to lack of effect on tendon itself [65]. Such data suggest that there may be a fine line between the osteogenic and tendon promoting activity of BMP2. BMP7 (identical to OP1 growth factor in literature) likely plays an important role during embryonic development as it is strongly expressed in developing chicken tendons [66]. BMP7 is also a more potent promoter for cell proliferation and type I collagen synthesis in tendon cell cultures established from both young and aged male tendons than BMP2 which performs these functions well in cell cultures from young but not older tendons [67]. As such BMP7 might be more suitable for clinical use as a tendon healing agent.

As pointed out by Lincoln et al. heart valves and musculoskeletal system, including tendons share regulatory mechanisms [30]. The BMP2-Sox9-aggrecan axis is an example of a pathway connecting regulation of cardiac and skeletal development. This pathway is instrumental in endocardial cushion formation, and in the differentiation of chondrogenic cell lineages [30, 68]. Transcription factor Sox9 regulates expression of chondrogenic genes and their protein products such as type II and type XI collagens, aggrecan, cartilage link protein and some ECM proteins produced in chicken heart valves [68–72]. It is interesting that aggrecan-expressing cartilage like cells have been found in the spongiosa layer of heart valves. The spongiosa layer is sandwiched in the middle between the fibrosa and atrialis layers where it exhibits compressibility, similar to a layer of cartilage [73].

On the other hand, it is the FGF-scleraxis-tenascin which regulates the development of semilunar valve precursor cells into valve cups and chordae tendineae (the "tendons" of heart valves), and its expression is equally important during tendon cell lineage differentiation [30, 74].

5.3 Fibroblast Growth Factors

Like most other families of growth factors, fibroblasts growth factors (FGFs) participate in embryonic development, more specifically, they regulate gastrulation, neurulation, anteroposterior specification of body segments and organ morphogenesis [75, 76]. In postnatal life they function as classical growth factors, including their function as powerful promoters of angiogenesis. This group consists of at least 23 growth factors binding to four high-affinity transmembrane receptors with tyrosine kinase activity (FGFRs). The receptors are subject to alternative splicing which modulates their activity [77, 78].

Several FGFs are involved in myogenesis which is directly associated with tendon development during the embryonic stage [79, 80]. FGF2, known previously as basic FGF, is synthesized and secreted without a signal peptide. It is a potent mitogen, and a potent angiogenic agent as well [22, 78]. It also induces regeneration of the chicken limb bud after application of FGF2-loaded heparin gel beads to the amputation site [81]. FGF2 directed development of tenocytes from multipotent mouse cells [82], and stimulated proliferation of mouse tendon cell lines together with induction of scleraxis, a transcription factor and an early tendon marker [83]. Ide et al. have shown that FGF-2 accelerated initial tendon-to–bone healing due to stimulation of formation of tendon and fibrocartilage tissues but not bone, something what BMP2 failed to provide [84].

FGF4, unlike FGF2, is synthesized with a signal peptide which is cleaved off after extracellular secretion [85, 86]. Though originally discovered as an oncogene, FGF4 is expressed in preimplantation mouse blastocysts. Its multiple roles during embryonic development include facilitation of epithelial-mesenchymal communication (vital for morphogenesis) and maintenance of self-renewal and pluripotency of stem cells. More

pertinent to the topic under discussion here is that FGF4 also promotes early cardiac development [77], limb development and, as already described above, expression of scleraxis, [30, 77]. Scleraxis in turn induces expression of tenascin, another tendon marker. Both scleraxis and tenascin are also expressed in valve progenitor cells derived from chicken embryos [73].

Their avid binding to heparan sulfate and other glycans present in the extracellular matrix enables FGFs to be stored in the extracellular matrix and to be readily available for use in tissue repair. Whether FGFs are suitable for accelerating or improving healing of tendon injuries in clinical setting remains to be evaluated because their potent angiogenic capacity may interfere with proper assembly of collagen fibrils, especially in the rather avascular tendon and ligament tissues (and in the avascular cardiac valves as well). The angiogenic ability of these growth factors spurred experimental application of recombinant FGF1, FGF2 and FGF4 gene therapy aimed at treatment of cardiovascular problems though clinical use is still far away in the future [78].

5.4 Role of IGF-I in Tendon Healing

Unlike the huge TGFβ superfamily, the IGF family has only two members: IGF-I and IGF-II, both of which retain their close relationship to insulin as they evolved from an ancestral insulin-like gene by duplication [87]. IGF-I has been much better characterized and so it is the one under discussion in this review. It is mainly produced by the liver under the control of growth hormone (GH) [88]. It has several important roles, mediation of growth hormone activity is one, stimulation of proliferation of various cell types, of animal and human origin, including tenocytes is another one [22, 89, 90]. It can bind to any of the six IGF binding proteins which are also synthesized in the liver and their main function is to regulate IGF-I activity [22, 91, 92]. IGF-I receptor bears partial homology to insulin receptor and c-ros oncogene. The mature form of this receptor is a heterotetramer $\alpha_2\beta_2$ of 350 kDa which upon ligand binding leads to activation of tyrosine kinase [93, 94]. Tsuzaki et al. have found IGF-I mRNA, IGF-I and receptors for IGF-I in both tendon surface cells (residing in the epitenon) and tendon internal fibroblasts (embedded in collagen bundles and fascicles), and they noted an increase in the levels of IGF-I and its mRNA in actively proliferating cells [90]. Hansson et al. have observed the induction of IGF-I synthesis in tendon cells after injury and with mechanical loading [95, 96]. IGF-I is known to stimulate collagen synthesis in fibroblasts, including tenoblasts [22, 97] which is closely associated with and dependent on mechanical loading of the tendon [97–99]. It is not clear whether IGF-I stimulates collagen directly through the PI-3 kinase pathway alone [100] or through the PI-3 kinase and ERK complex [101]. Some studies even indicate that IGF-I stimulates collagen production indirectly through TGFβ1 [102]. In any case, increased collagen synthesis in tendons appears to be a part of a systemic response to exercise, especially to repeated and sustained training in people. Regular exercise leads to sustained elevation of blood levels of GH and IGF-I due to an increase of GH release from the pituitary, and this is accompanied by increased expression of IGF-I mRNA in human skeletal muscle and tendon [99]. However, it remains speculative whether there is a causal relationship between elevated circulating GH/IFG-I and stimulation of collagen synthesis in the musculoskeletal system [99] as other investigators have not seen stimulatory effect of GH on tendon or ligament healing at least in some animal models [103, 104]. The stimulatory effect of IGF-I alone (i.e., without involvement of GH) in these models suggests only a local rather than systemic effect of IGF-I. Similarly, Yamaguchi et al. showed that loading-induced IGF-I expression in skeletal muscle occurs independently of release of GH from the pituitary [105]. Banes et al. have shown that mechanical load alone did not stimulate DNA synthesis, but only when both IGF-I and PDGF-BB were added to cultures of avian tendon cells [98].

The presence of several isoforms of IGF-I, the result of alternative slicing of IGF-I pre-RNA, compensates for the smallness of the IGF family and helps explain the role of IGF-I in stimulation of collagen synthesis in the tendon as a consequence of mechanical loading [106]. So far, three isoforms of IGF were identified: IGF-IEa, IGF-IEb and IGF-IEc. Rat isoform IGF-IEb is identical to human IGF-IEc, and to mechano-growth factor (MGF) which got its designation because of its association with mechanical loading in the muscle and tendon [106]. MGF, unlike standard IGF-I, is a strictly autocrine/paracrine factor produced locally upon proper stimulation [107]. Upregulation of MGF and IGF-Ea 7 has been observed in the Achilles tendon after eccentric and concentric training [108]. However, and somewhat paradoxically, prolonged hindlimb suspension also led to increase in MGF mRNA in rat Achilles tendon [109].

One can only speculate that the application of MGF could alleviate tendon problem and joint laxity in patients with Ehlers-Danlos syndrome, cutis laxa and related disorders.

IGF-I binding proteins (IGF-BPs) are involved in tendon healing as well, though their specific roles have not been yet established. Whereas the levels of mRNA for IGF-I and IGF-BPs 2–4 increased following injury, especially later, at 4 weeks after injury [35], those for IGF-BP 5 and 6 decreased [110]. Both IGF-I and IGF- BP4, and their respective mRNAs were co-localized to tendon fibroblasts and vascular endothelium in the human Achilles tendon [111]. The increase in IGF-BP 4 level seen in the Achilles tendons during long distance running can be attributed to an increase in mechanical loading [111].

The outcome of rather common tendinopathies and injuries of superficial digital flexor and other weightbearing tendons in racehorses is not satisfactory when only more traditional treatment and management are applied [112–114]. Though initial studies suggested that local administration of IGF-I enhances collagen synthesis in equine tendon explants [115] and improves equine tendon healing in vivo [112, 116, 117], more recent reports were less enthusiastic about the long-term prognosis of IGF-I-treated tendon injuries in racehorses [118].

5.5 PDGF

Growth factors, such as PDGF, and VEGF, have been shown to be synthesized and likely to play similar roles in tissue repair [119], though perhaps not in tendon healing. These two growth factors, or rather these two families of growth factors share 20 % homology of their respective amino acid sequences [22]. Like many other growth factors, PDGF/VEGF growth factors contain cysteine knots in their molecules [120].

PDGF is instrumental in repair mechanism of vasculature [121]. It is stored in platelet α granules so it gets released and ready for action during wounding or bleeding when restoration of vasculature becomes essential. PDGF is synthesized by endothelial cells, placental trophoblasts, smooth muscle cells, macrophages and sarcoma cells. PDGF is a 29–33 kDa glycoprotein, usually composed of two chains (at least in humans), either A or B which are joined by disulfide bridges. There are several other isoforms, but heterodimer PDGF-AB and homodimers PDGF- AA and PDGF-BB are the best described. PDGF and its isoforms bind to two types of receptors with five extracellular immunoglobulin loops and with an intracellular tyrosine kinase domain. PDGF is required by fibroblast, smooth muscle and glial cells for their optimal growth and cell proliferation. Its role in wound healing and as a chemotactic agent for fibroblasts and smooth muscle cells, neutrophils and macrophages has been well documented. Besides its involvement in blood vessel repair it is involved in the pathogenesis of atherosclerosis, pulmonary fibrosis and glomerulonephritis [22]. PDGF-AA and PDGF-BB are important mitogens for normal and neoplastic mesenchymal tissues. For example, exogenous PDGF-BB had a modest effect on healing of equine superficial digital flexor tendons [122] and of rat Achilles tendons [123]. PDGF cooperates with other growth factors. The classic paradigm is that while PDGF renders cells “competent” to replicate their DNA, and thus prepares cells for IGF-I activity as the actual growth factor moving cell through the cell cycle into the S phase when cells start synthesizing DNA [124]. This explains

why, as described above, avian tendon cell cultures required both growth factors in addition to mechanical loading for DNA synthesis [98]. The excellent review by Andrae et al. provides more information on all aspects of PDGF biology and role in physiology [121].

PDGF delivered via in vivo gene transfection is capable to enhance healing of rat Achilles tendons as determined by histological and biomechanical parameters. The gene transfer was superior to direct administration to the tendons [125]. PDGF alone, in particular the homodimer PDGF- BB, or in combination with IGF-I stimulated regeneration of periodontal ligament and its attachment to the alveolar bone [126], a matter of importance in Marfan patients with periodontitis. By the way, FGF2, discussed in more detail above, had similar effect on cells of the periodontal ligament [6]. PDGF was shown to improve healing and biomechanical parameters of acute injury of the medial collateral ligament in rabbits and rats, especially, if administered shortly after injury. [127, 128].

5.6 VEGF

VEGF and its isoforms bind to three receptors, VEGF-R1-3, which belong to a subfamily of tyrosine-kinase receptors within the PDGF-receptor class. Just like PDGF, VEGFs are composed of two subunits linked by disulfide bonds [14] These growth factors are usually considered exclusive angiogenic factors, particularly VEGF-A [22]. VEGF-A (designated just as VEGF for the sake of simplicity from now on) is the main VEGF representative [22]. More recent studies have shown that VEGF has the ability to function as an autocrine mitogen for cancer cells [129].

Whereas splice variants of VEGF, $VEGF_{121}$ and $VEGF_{165}$, have been identified in fetal tendons, healthy adult tendons possess little VEGF [14]. Instead, adult tendons express endostatin, an antiangiogenic peptide which downregulates VEGF-induced Erk1/2 kinase phosphorylation [130, 131]. VEGF presence and neovascularization appearance in the tendon are signs of tendon overuse injuries [14], and they impede rather than stimulate proper healing of tendons [132, 133]. This is due to a sequence of several events. Injury to rat patellar tendon results in hypoxia, and in increased expression of hypoxia-inducible factor-1 (HIF-1) which in turn induces transcription of VEGF gene [134]. In addition to hypoxia, mechanical overload, an important factor in overuse tendon injuries, is another stimulator of VEGF synthesis [14] as is the production of epidermal growth factor and inflammatory cytokines (IL-1β, IL-6, IL-8) [14, 133]. To facilitate angiogenesis VEGF stimulates the production of matrix metalloproteinases (MMPs), enzymes actively degrading connective tissues so to assist sprouting of new blood vessels. VEGF also inhibits the production of tissue inhibitor metalloproteinases (TIMPs), enzymes counteracting MMP activity [14, 132, 135]. It is likely that VEGF regulates the production of MMPs and TIMPs by endothelial cells in blood vessels of injured tendons as well as by tenocytes as those latter cells express VEGF-R2 [136]. Increase and decrease in MMP-3 synthesis correlates well with timing of increase in VEGF synthesis at 7 days post-injury, and with decrease in VEGF synthesis 14 and 28 days post-injury, respectively [132]. And MMP-3 is not the only metalloproteinase associated with VEGF production. For example, expression of MMP-1 and MMP-13 is also regulated by VEGF, at least in chondrocytes [14]. MMP-9 secreted by inflammatory cells follows the same cycle. It is elevated early in tendon healing, its decrease 7 days after wounding is associated with rapid increase first in type III collagen, which is followed by an increase in type I collagen. MMP-2 and MMP-14 were found to be elevated 21 days after wounding when granulation tissue starts undergoing extensive remodeling into organized collagen tissue, and scarring [137]. Increased or decreased levels of many MMPs were found in patients with ruptured rotator cuff, supraspinatus, Achilles and other tendons [138, 139]. The induction of MMP activity and angiogenesis by VEGF then leads to impaired biomechanical parameters of tendon due to matrix degradation [132]. These tissue changes also lead to pain, especially, in the case

of neovascularization, in mid-portion of chronic Achilles tendinosis [14, 140]. It is interesting that as Cook and Ramirez describe in the Chap. 6 on Clinical, Diagnostic, and Therapeutic Aspects of the Marfan Syndrome, inhibition of MM-2 leads to improvement in medial degeneration of aorta in a mouse model.

Another group of proteinases, members of a-disintegrin-and-metalloproteinase-with-thrombospondin-like-motifs (ADAMTS) activity family or aggrecanases are expressed in tendons as well [2, 139]. For example, upregulation of ADAMTS-4 was observed in injured tendons and in tendon cell cultures exposed to TGFβ, whereas no differences in ADAMTS-1 levels were noted between normal and diseased tendons [141] . Because expression of ADAMTS forms is tied with degradation of aggrecan they are discussed in more detail in Chap. 4 of this volume.

5.7 Other Modalities

That combinations of several growth factors have been found beneficial to tendon healing should not surprise anybody [36]. Not only such preparations are able to stimulate cell proliferation they also lead to improvement of mechanical properties of tendons [36, 142–145]. Such findings have been essential in tissue engineering where the current immediate goal is to improve cellular repopulation of acellularized scaffolds [143].

Rather than using purified recombinant or natural IGF-I or mixture of other growth factors,, a rather expensive undertaking, to treat tendon injuries, investigators have been using platelet-rich plasma (PRP) in experiments, animal trials and treatment of chronic leg ulcers, and in tissue engineering among other applications [146]. The use of PRP provides several advantages – it is rich in numerous growth factors, it provides other, some of them still uncharacterized compounds promoting tissue repair, and it is much easier and cheaper to manufacture than individual growth factors. PRP stimulates cell proliferation and collagen synthesis in human tendon cell cultures [147]. A study in rabbits showed accelerated healing of a ruptured Achilles tendon accompanied by an upsurge in IGF-I expression [146]. The effect of autologous conditioned serum on tendon healing was comparable perhaps because the conditioned serum increased expression of several growth factors (bFGF, BMP12 and TGFβ1) in an in vivo model of rat Achilles tendon healing [37]. PRP, in combination with BMP2, even accelerated healing of bone-tendon injuries, with PRP improving tendon healing and BMP enhancing bone healing in rabbits with their Achilles tendon surgically separated from the bone insertion [148]. Similarly, PRP had beneficial effect on healing of porcine ligaments, resulting in improvement of several biomechanical parameters, such as load to failure, maximum load and stiffness. However, this effect was rather limited as it did not improve laxity, maximum tensile strength or linear stiffness [149]. In a study of PRP effect on healing of surgically transected tendon of Achilles in rats, local application of PRP led to increased expression of type I and III collagens, and to improved ultimate tensile strength [150].

PRP products have been used in human medicine, mostly for healing of athletic injuries [15, 151]. Unfortunately, the use of PRP has not met with much success in human patients. Why this is so is not quite obvious. For one, pathogenesis of human tendinopathies may be quite different from artificial tendinopathies induced under highly simulated conditions, or from Achilles tendon rupture in rat or rabbits; two, there is no standardization for PRP preparations; three, the content of growth factors, cytokines and other compounds may differ between human and animal PRP [152, 153]. It is also possible that the presence of TGFβ in PRP might vary from batch to batch, and, if present in higher concentrations, would lead to fibrosis, and thus to worse outcome [40]. Perhaps most importantly, PRP is used on injuries affecting a wide variety of tendons, ligaments and even muscle strains without much, if any prior clinical evidence of effectiveness [15, 151].

As noted above, not only the presence of specific growth factors, but also the milieu and mechanical parameters determine the final fate of tissue

differentiation. This is particularly the case with stem cells which can be induced to commit toward bone or tendon (or many other different) lineages with cell culture on properly engineered substrates [154]. These substrates when functionalized with type I collagen and fibronectin were able to direct bone marrow-derived stem cells toward tenogenic differentiation and to production of BMP2/Smad8. The authors speculate that BMP2 facilitates healing at the bone-tendon interface by its ability to regulate both osteogenic and tenogenic differentiation depending on the degree of stiffness environment and through stimulating signaling of other growth factors [155]. Tenogenic differentiation required the presence of type I collagen with fibronectin being optional. The substrate itself had to be within a narrow range of elastic moduli. An intermediate elastic modulus then stimulated production of BMP2/Smad8, and ultimately of scleraxis. Osteogenic development demanded stiffer substrate and fibronectin, type I collagen was inhibitory, especially with less stiff substrates [155]. It has also been shown that mechanical stimulation of sponge constructs in vitro modulates stiffness, and such constructs upon application in vivo enhance tendon repair, at least in rabbits [156].

It is likely that bioengineering of soft tissues will play an important role in treatment of disorders such as Marfan or Ehlers-Danlos. Replacement of diseased portion of aorta by bioengineered constructs would prevent the development and/or rupture of aortic aneurysm. Similarly, bioengineered tissues have a great potential to serve as patches in other organs with potential to rupture (e.g., uterus in women with Ehlers-Danlos), or as replacement for affected tendons in Ehlers-Danlos.

As noted above injuries of the superficial digital flexor and other weight bearing tendons are common in horses of all breeds, but particularly in horses of racing breeds. Because the full recovery and return to races is rather rare after a tendon injury, and because of financial considerations, such as investments into racehorses, the field of stem cell therapy for equine tendons has been quite active and has met with some success. Some investigators have identified progenitor cells residing in the superficial digital flexor tendon and found these cells comparable to bone marrow derived stem cells. The tendon-derived progenitor cells expand quickly in cell culture and express the same markers as bone marrow-derived cells. The advantage is that tendon-derived progenitor cells have high clonogenic properties and limited chondrogenic differentiation potential [157]. There is one caveat: though tendons have multipotent progenitor cells, likely residing in the endotenon between the collagen fascicles and vasculature, it is very difficult to activate such cells in vivo, especially in more mature tendons [158]. However, autologous bone marrow- or peripheral blood-derived stem cells are easier to obtain, and they have been used for treatment of equine tendon injuries for several years. Almost flawless repair of these tendons with fibers perfectly aligned parallel to the long axis of the tendon itself was achieved in three injured horses with a single injection of autologous "deprogrammed" peripheral blood-derived stem cells [159]. Three other horses with similar tendon injuries who served as controls and received traditional therapy ended up with tendon scarring (documented by ultrasound) and re-injury only a few months later [159]. Godwin et al. harvested mesenchymal stem cells from a bone marrow aspirate which was suspended in culture medium. The bone marrow-derived mesenchymal stem cells attached themselves to the plastic of the tissue culture vessels and proliferated until confluent. These cells were then detached from the plastic and implanted directly into the injury site of 141 racehorses [158]. As in the study of Marfe et al. [159], many horses were able to re-enter racing with low re-injury rate. The ultrasonic appearance indicated complete healing without any evidence of ossification in the healed tendon [158]. Similar results were achieved by other groups as well [160, 161], though treatment with bone marrow-derived stem cells had no effect on the diameter of collagen fibrils in the healing tendon [162]. Equine adipose-derived stromal vascular fraction was particularly effective in stimulation cell proliferation, and application of equine cord blood-derived mononuclear cells led to increased production of interleukin 6, TGFβ1 and prostaglandin E_2 at least in vitro [161], though Uysal

et al. have shown the effectiveness of adipose-derived stem cells on healing of rabbit primary tendon healing in vivo [163].

5.8 Epilogue

Though we do have a better understanding of the mechanism of tendon injury and subsequent repair, we still have a long way to go before we will feel confident that we have master the art of tendon healing. Progress in growth factor and stem cell research is bringing us closer not only to the development of new strategies for treatment of tendon injuries, but also to better understanding of pathogenesis of heritable diseases of soft tissues. What we have to do now is to figure out how to use our knowledge to improve the lives of patients afflicted with these disorders.

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Clinical, Diagnostic, and Therapeutic Aspects of the Marfan Syndrome

6

Jason R. Cook and Francesco Ramirez

Abstract

Marfan syndrome (MFS) is a relatively common and often lethal disease of connective tissue. Medical, surgical and basic research advances over the last two decades have had a major positive impact on the clinical management of MFS patients. Life expectancy has increased significantly, more discriminating diagnostic criteria have been developed, a number of new clinical entities have been recognized, and exciting opportunities for drug-based therapy have emerged. Despite such a remarkable progress, MFS diagnosis remains difficult and aortic disease progression is very heterogeneous and clinical outcome is unpredictable. Ongoing research efforts are therefore exploiting animal models of MFS to identify novel diagnostic and prognostic biomarkers, genetic, epigenetic and environmental modifiers and druggable biological targets.

Keywords

Marfan syndrome • Mutations in gene for fibrillin-1 (*FBN1*) • Thoracic and abdominal aortic aneurysm • Valvulopathy • Ghent nosology • β-blockers • Calcium channel blockers • Angiotensin receptor blockers (ARBs) • Bone deformities

6.1 Introduction

Marfan syndrome [MFS; Online Mendelian Inheritance in Man (OMIM) #134797] is a relatively common multi-system disease that exhibits high penetrance and marked inter- and intrafamilial variability [1]. First described in an 1896 case report by Antoine-Bernard Marfan, MFS was subsequently identified by Victor McKusick as the archetypal "heritable disorder of connective tissue" [2, 3]. McKusick also hypothesized that the pleiotropic manifestations of MFS may reflect

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structural or metabolic dysfunctions of an extracellular matrix (ECM) component. This prediction was eventually validated in 1991 with the identification of causative mutations in *FBN1*, the gene encoding the ECM protein fibrillin-1 [4, 5]. This seminal discovery, together with improved delineation of the phenotype, has resulted in stringent diagnostic criteria that more effectively distinguish MFS from clinically related conditions with different genetic lesions, repertoire of manifestations, natural history, and response to treatment [6, 7]. Similarly, advances in the surgical management of cardiovascular complications have substantially extended life expectancy in MFS patients [8]. Lastly, creation of mouse models of MFS has provided invaluable insights into disease-causing mechanisms that are being translated into new pharmacological interventions [9]. Clinical, diagnostic and treatment aspects of MFS are discussed in this chapter along with recent experimental findings relevant to the pathogenesis and therapy of organ-specific manifestations in this heritable disorder of connective tissue.

6.2 Clinical Phenotype

MFS is a pleiotropic disease with predominant manifestations in the cardiovascular, skeletal, and ocular systems; additionally, the skin, fascia, lung, skeletal muscle and adipose tissue may be involved [1]. MFS can manifest either at birth with a significantly dilated aorta that dissects and ruptures within the first months/years of life, or as a progressive disease that can be diagnosed as late as 30-40 years of age when the majority of fatal events usually occur in untreated patients [1, 10–14]. Extensive phenotypic variability, age-dependent onset of informative manifestations and high degree of spontaneous mutations however limit MFS diagnosis and management, particularly in children and adolescents [1].

6.2.1 Cardiovascular System

Cardiovascular abnormalities affect more than 80 % of MFS patients with severe consequences for fitness and survival [15, 16]. Common cardiovascular manifestations include enlargement of the aortic root and proximal ascending aorta, which often precipitate dissection and rupture of the vessel wall, pulmonary artery dilatation, which rarely dissects, and myxomatous valve changes, which can be associated with insufficiency of the mitral and aortic valves and progressive myocardial dysfunction.

Aortopathy: Dilatation of the aortic root frequently begins in utero, can be detected neonatally by echocardiography, and progresses at a heterogeneous and unpredictable rate [17–21]. Age-dependent histomorphological changes of the MFS aorta, sometimes improperly referred to as cystic medial necrosis, are associated with a stiffer vessel and include elastic lamellae fragmentation and disorganization, increased collagen and mucopolysaccharide accumulation, and a relative paucity of vascular smooth muscle cells (vSMCs) [22–24]. Other elastic arteries may also display medial degeneration but dilatation is uncommon, except in the pulmonary artery [25, 26].

Aortic root diameter is measured at the sinuses of Valsalva and normalized against age and body size (i.e.: body surface area; BSA); such a measurement is however complicated by the relatively greater BSA of MFS patients compared to unaffected individuals [7, 27]. While aortic size and elastance also increase in the general population as the result of aging and/or hemodynamic stress, these features progress significantly more rapidly in MFS patients; moreover, MFS patients dissect at a smaller aneurysm size than other individuals. Accordingly, prophylactic surgery is normally recommended when the diameter of the aortic root reaches ~5 cm, unless there is a family history of early dissection or dilatation progresses with unusual rapidity (>0.5–1 cm/year) [28].

While thoracic aortic aneurysm (TAA) is a major finding in MFS, abdominal aortic aneurysms (AAA) may also occur more typically in MFS patients who have undergone repair of the proximal aorta [29, 30]. In a few instances, MFS patients may also display AAA out of proportion to proximal aortic disease. Similar to TAA, AAA in MFS tends to dissect at a smaller diameter

than in non-MFS patients. AAA is a common manifestation associated with aging and environmental triggers, such as atherosclerosis and smoking [31–33]. Arterial stiffness is an independent risk factor for AAA dissection and increased stiffness in MFS occurs throughout the arterial tree [34–36].

Pulmonary artery dilatation: Dilatation of the pulmonary artery in MFS is rarely associated with dissection owing to the lower mean pressure within this vessel (>20 mmHg) compared to the ascending aorta (>80 mmHg) [26]. According to the LaPlace equation, tension in the vessel wall is proportional to the pressure within the vessel times the radius of the vessel divided by wall thickness. It follows that tension in the wall of the pulmonary artery is significantly less than in the ascending aorta, even though the aortic wall is slightly thicker, and increased systolic pressure produces greater tension thereby promoting aortic dissection. Dilatation of the pulmonary artery in MFS therefore suggests an underlying defect in vasculogenesis independent of hemodynamic pressure.

Valvulopathies: Multiple valvular abnormalities are frequently seen in MFS; they include myxomatous thickening with prolapse and regurgitation of the mitral and tricuspid valves, and insufficiency of the aortic and pulmonary valve leaflets [9, 37]. In contrast to the other valvular abnormalities, mitral valve prolapse (MVP) can have a major impact on cardiovascular function. Increased length and physical alterations of the mitral valve lead to regurgitation during systole, in association with precordial systolic murmur. MVP prevalence in MFS is ~75 % with 25 % of the cases manifesting myxomatous valve thickening [38–40]. By contrast, prevalence of MVP in the general population is about 1.3 % [41]. Although a common trait of adult MFS patients, neonatal MVP with severe mitral regurgitation can precipitate cardiac dysfunction and congestive heart failure when untreated. [42–44] Moreover, mitral valve repair can occasionally lead to TAA dissection and rupture due to acute changes in cardiac function and pressure [45].

Cardiomyopathy: Several studies of cardiac function in MFS have reported an increase in the size and mass of the left ventricle (LV) along with systolic and diastolic dysfunction [14, 46–49]. While it is generally believed that MVP with severe mitral regurgitation is the main determinant of LV and left atrial dysfunction, the occasional finding of dilated cardiomyopathy (DCM) in the absence of valvular disease has raised the possibility of a primary myocardial insufficiency in MFS [13, 14, 17, 49–51]. The relative abundance of fibrillin-1 assemblies in the myocardial matrix indirectly supports this hypothesis. Another untested possibility is that the stiffer aortic wall of MFS patients may contribute to cardiac dysfunction, by itself or in combination with valvular disease [52].

Whether or not spontaneous DCM is part of the MFS phenotype remains controversial due to conflicting studies of myocardial performance in affected patients and the lack of relevant data from mouse models of the disorder [17]. As a result, closer monitoring of heart function has yet to become the standard management of cardiovascular manifestations in MFS and no new pharmacological strategies are currently being investigated to curb DCM formation in this condition. This is a particularly important issue for patients with severe neonatal presentation of MFS who are at a significantly higher risk of succumbing to heart failure.

6.2.2 Skeletal System

The most striking and immediately evident MFS pathologies involve disproportionate linear overgrowth of tubular bones and ligament laxity, which promote malformations of the digits (arachnodactyly), limbs (dolichostenomelia), spine and anterior chest wall [6, 53]. Craniofacial deformities, dural erosion of bony tissue (dural ectasia), osteoarthritic changes secondary to prolonged protrusion of the femoral head (protusio acetabuli), hindfoot valgus with forefoot abduction and lowering of the midfoot (pes planus), and decreased bone mineral density (osteopenia) are additional skeletal findings in MFS [28, 54]. Craniofacial abnormalities include a long narrow skull (dolichocephaly), arched palate with teeth

crowding, recessed lower mandible (retrognathia) and orbital sockets (endopthalmos), downslanting palpebral fissures, and reduced cheek bone size (malar hypoplasia). Ligament/tendon laxity carries an increased risk of musculoskeletal injury through destabilization of joints (e.g.: knee and ankle sprains). Skeletal manifestations in MFS, albeit common, are the least sensitive diagnostic criteria due to considerable prevalence of these traits in the general population as well as in individuals afflicted with other connective tissue diseases. An important exception is the highly diagnostic "thumb and wrist" sign, which reflects the combined outcome of increased digits length (arachnodactyly) and ligament laxity [6].

Skeletal malformations can negatively impact MFS fitness, particularly in older individuals where late-onset complications are an emerging medical problem. Osteopenia is a case in point as inadequate protocols to compare bone mineral density (BMD) between affected and healthy individuals and lack of robust normative data for children hamper the ability to predict the risk of fractures [55, 56]. Pain is another poorly managed aspect of the MFS phenotype. Secondary manifestations of dural ectasia include low back pain, headache, proximal leg pain, weakness and numbness above and below the knee, and genital/rectal pain [57, 58]. Spine and chest deformities are a major morbidity factor in MFS for they cause sternal protrusion or depression (pectus carinatum and pectus excavatum, respectively), vertebral displacement and severe spine deformities in the form of scoliosis (lateral spine displacement) and/or lordosis and kyphosis (anterior and posterior spine displacements, respectively) [59, 60]. Moreover, a positive and significant correlation has been reported between aortic root dilation and heightened body growth during infancy and adolescence [61]. Surgical repair of severe chest deformities is sometimes required to improve cardiac and pulmonary function or increase the surgeon's ability to repair the ascending aorta [62]. These interventions are commonly performed after MFS patients reach skeletal maturity as to avoid the recurrence of chest abnormalities due to continued rib overgrowth. Bracing is usually inadequate to manage severely progressive scoliosis, and surgical repair carries significant risk of complications [59].

6.2.3 Ocular System

Major ocular abnormalities in MFS include lens dislocation (ectopia lentis), myopia and retinal detachment with the first manifestation being the most common occurrence (60 % of patients) [63]. Ectopia lentis is usually bilateral and non-progressive, and may range from asymptomatic displacement to significant sublaxation. Glaucoma and premature cataracts are recognized complications of severe ectopia lentis [64]. Refractive aids, pharmacological manipulation, and lensectomy are frequently employed to improve vision. Ocular globe elongation leading to myopia (nearsightedness) is the second most common ocular abnormality of MFS (~40 % incidence) [65, 66]. Albeit less frequent than other ocular manifestations (8 % frequency), retinal detachment is the most serious complication, generally manifesting in the mid-20s and often involving both eyes [67, 68]. The frequency of retinal detachment increases to ~23 % in MFS patients who manifest ectopia lentis. Retinal repair is challenging, particularly in young patients; however, new surgical techniques and instrumentations, together with more effective tools for early detection of ocular problems, have significantly improved the care of the MFS eye.

6.2.4 Other Organ Systems

Spontaneous pneumothorax, apical blebs, and bullous emphysema are lung abnormalities that can be associated with MFS [69–72]. Spontaneous pneumothorax, in particular, is considerably more frequent in MFS patients than in the general population and is thought to result from the rupture of an apical bleb [73]. Similar to the problems connected with normalizing TAA and BMD measurements, normalization of pulmonary function tests can frequently be confounded by the greater than the average limb-to-thorax length of MFS patients [74]. Cutaneous stretch marks

(striae atropicae) are commonly found on the axilla, arms, flanks, hip, mid and lower back of afflicted individuals [75, 76]. In contrast to the typical stretch marks associated with weight loss or pregnancy, those in MFS involve areas of flexural stress and may therefore reflect a mechanically impaired integumental matrix. MFS often display a myopathic appearance with little muscle mass that fails to increase in response to growth and exercise [76]. Individuals with neonatal onset of severe and rapidly progressive MFS have profound skeletal muscle hypoplasia and hypotonia throughout life. Adipose tissue deficiency is an anecdotal finding, particularly in MFS children and adolescents. However, several MFS patients have considerable muscle and adipose mass from an early age.

6.3 Diagnostic Criteria

Despite the seminal identification of the underlying genetic defect, MFS remains a clinical diagnosis that cannot be validated by a single molecular test. Diagnostic criteria originally published in the so-called Ghent nosology include major and minor criteria, organ involvement, and combined manifestations constituting major or minor criteria (Table 6.1) [66]. Specifically, the presence of one major criterion and involvement of an additional organ system are required for positive diagnosis in cases of documented family history or harboring a bona fide MFS (*FBN1*) mutation; in all other instances, major criteria in two different organ systems and involvement of a third organ system are required. A revised Ghent nosology has been published more recently that places a greater emphasis on aortic root enlargement/dissection, lens dislocation and MFS-causing *FBN1* mutations (Table 6.2) [28]. Accordingly, aortic root enlargement/dissection and lens dislocation are sufficient for MFS diagnosis even in the absence of any family history, whereas a bone fide *FBN1* mutation or a combination of systemic manifestations is required in the presence of only one of these cardinal MFS features. Systemic manifestations are scored according to a numerical matrix of individual traits in which a total score ≥ 7 indicates systemic involvement [28]. Age-dependent onset of informative manifestations, particularly aortic root diameter, is a widely recognized problem in diagnosing young MFS patients with or without family history or disease-causing *FBN1* mutations. These individuals are therefore considered as being affected by a "non-specific connective tissue disorder" (in the absence of family history) or by "potential MFS" (in the presence of a *FBN1* mutation) and should be subject to regular follow-up until aortic growth reaches the threshold score of $Z \geq 3$ [39].

While the revised Ghent criteria are anticipated to decrease the number of false-positive diagnoses, two important issues remain that make MFS nosology a work in progress [77, 78]. As already alluded, normalization of aortic root diameter against BSA to generate an informative Z-score can be problematic with both pediatric and adult patients. The proposal of using a multivariate formula that takes into account potentially confounding criteria, such as age and gender, may ameliorate this problem [77]. Similarly, future review of the systemic score will validate whether the proposed ≥ 7 systemic point threshold is indeed indicative of a major criterion.

6.4 Management and Treatment

Lifestyle modifications, regular echocardiographic assessment, pharmacological treatment and prophylactic surgery are the major tools currently used to manage the life-threatening cardiovascular complications of MFS. Reducing emotional stress, which may raise heart rate and blood pressure, and restricting physical activities, which may increase the risk of TAA rupture, are strongly emphasized [79]. MFS patients who wish to exercise are therefore recommended isotonic low-impact sports, such as swimming or biking, that reduce blood pressure and heart rate.

Serial echocardiographic imaging of the aorta is imperative due to the heterogeneous rate of aneurysm progression. Once diagnosed, patients are evaluated more frequently to establish baseline changes in aortic root dilatation [29]. Current

Table 6.1 Ghent nosology

System	Major criteria	Minor criteria
Cardiovascular	Ascending aortic dilation (involving sinus of Valsalva)	MVP
	Aortic dissection	Dilatation of the main pulmonary artery (<40 years)
		Calcification of the mitral annulus (<40 years)
		Dilatation or dissection of descending thoracic or abdominal aorta (<50 years)
Ocular	Ectopia lentis	Flat cornea
		Elongated globe
		Hypoplastic iris
Skeletal	At least 4 of the following:	Moderate severity pectus excavatum
	Pectus carinatum or pectus excavatum	Joint hypermobility
	Reduced upper to lower ratio	Highly arched palate
	Arm span to height ratio >1.05	Facial appearance
	Wrist and thumb sign	Dolichocephaly
	Scoliosis of >20 or spondylolisthesis	Malar hypoplasia
	Reduced ext. of elbows (<170)	Enophthalmos
	Medial displacement of mdial malleolus	Retrognatia
	Protrusio acetabulae	Down-slanting palpebral fissures
Pulmonary	None	Spontaneous pneumothorax
		Apical bleb
Skin	None	Striae atrophicae not associated with weight changes, pregnancy or stress
		Recurrent or incisional hernia
Nervous	Lumbosacral dural ectasia by CT or MRI	None
Family/genetic history	Having a parent/child/sibling who meets these diagnostic criteria	None
	Presence of a mutation in *FBN1* known to cause MFS or haplotype around *FBN1* inherited by descent and associated with familial MFS	

guidelines suggest that pediatric patients or patients with accelerated aortic root growth should be evaluated twice rather than once a year. The same consideration applies for patients who have undergone aortic root, ascending aorta, and/or arch replacement. As aneurysms can form distal to the site of surgical repair, annual CT or MRI scans should be employed to properly visualize the entire aorta [30].

Propranolol, a non-selective β-adrenergic antagonist (β-blocker), has been the mainstay MFS treatment since the mid-1990s [80–82]. Propranolol and other β-blockers are commonly prescribed to treat hypertension due to the ability of reducing cardiac output and peripheral vascular resistance and consequently, tension on the aorta. Although β-blockers are standard MFS care, data documenting the therapeutic benefits of these drugs are relative few and often controversial, particularly in treating MFS children [82, 83]. β-blockers are also used to prevent aortic complications in pregnant MFS women and to stabilize MFS patients with acute aortic dissection. Calcium channel blockers are

Table 6.2 Revised Ghent nosology

	Cardinal features (Need 1 of 3)	Systemic features (≥7 pts)
Cardiovascular	Aortic root Z-score > 2	Mitral valve prolapse (1 pt)
Ocular	Ectopia lentis	Myopia > 3 diopters (1 pt)
Skeletal		Wrist AND Thumb Sign (3pts)
		Wrist or Thumb Sign (1 pt)
		Pectus carinatum (2 pt)
		Pectus excavatum/chest asymmetry (1 pt)
		Protrusio acetabuli (1 pt)
		Reduced upper span to lower span ratio AND increased arm/height ratio with no scoliosis (1 pt)
		Scoliosis or thoracolumbar kyphosis (1 pt)
		Reduced elbow extension (1 pt)
		Facial features (3 or 5) (1 pt)
		Dolichocephaly
		Malar hypoplasia
		Enophthalmos
		Retrognatia
		Down-slanting palpebral fissures
Pulmonary		Pneumothorax (2 pt)
Skin		Skin striae (1 pt)
Nervous		Dural ectasia (2 pt)
Family/Genetic history	*FBN1* mutation identified in an individual with MFS	

prescribed as second-line therapy for aortic complications in the 10–20 % of MFS patients who do not tolerate β-blockers. The treatment is supported by the positive outcomes of an early prospective study of six MFS patients treated with verapamil, and a very recent clinical trial comparing the impact of β-blockers and calcium channel-blockers on vascular function and central aortic pressure in sixteen MFS patients [84, 85]. On the other hand, antibiotic prophylaxis is strongly recommended whenever transient bacteremia may occur (e.g.: during dental cleaning) in MFS patients with documented valvular regurgitation and are therefore at a greater risk of endocarditis [86].

As described more extensively later, mouse models of MFS have revealed new therapeutic opportunities for TAA; principle among them is the use of drugs that target different components of the renin-angiotensin system, such as angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEi). Based on mouse findings, a non-randomized retrospective analysis of 18 pediatric MFS patients refractory to ACEi and β-blockade has documented appreciable decrease in aortic root growth after therapy with the ARB losartan was initiated [87]. Ongoing prospective clinical trials using larger cohorts of MFS patients will test the validity of this retrospective study [88–91]. In addition to ARB therapy, a single prospective study with MFS patients has documented that beneficial effect of the ACEi enalapril on reducing aortic root growth and aortic stiffness versus propranolol or atenolol [92]. Although ARBs are better tolerated than ACEi, both classes of medications are contraindicated during pregnancy due to teratogenicity; consequently, β-blockers are the only suitable therapy to prevent TAA dissection in pregnant women with MFS.

Mouse studies have also implicated inflammatory cells and matrix metalloproteinases (MMPs) in the process of medial degeneration that accompanies TAA progression in MFS [93, 94]. Inhibiting the activity of MMP-2 and -9, through doxycycline, or inflammatory cells, through statins, were both found to mitigate TAA progression in MFS mice [95–97]. A similar phenotypic improvement characterizes MFS mice lacking MMP2 and was associated with a decrease in improper TGFβ signaling [98]. Additionally, cell culture studies have shown that proteolytic fibrillin-1 peptides up-regulate MMP-1 expression [99]. Some of these findings have independently been validated in human patients by histological analyses of aortic specimens and abnormally high inflammation markers in plasma [100, 101]. On the other hand, MMP inhibition in AAA patients via administration of either doxycycline or statins has yielded controversial results [102, 103].

Although improved procedures to replace the diseased aorta have significantly reduced post-surgical complications in MFS to below 2 %, emergency surgery for acute dissection still carries a high degree of mortality (>10 %) [8]. The Bentall and De Bono procedure has been used for several years to repair the MFS aorta by replacing the aortic valve, root and ascending portion with a Dacron graft valve [104]. While the mortality associated with the Bentall and De Bono procedure is relatively low, replacement of the aortic valve with a mechanical valve requires patients to be on anticoagulant medications in increases the risk of endocarditis [105, 106]. A recent adaptation to simply replacing the aortic root and valve is to remodel the Dacron graft to reproduce the aortic sinuses (Yacoub or David II procedure) with the re-implantation of the patient's aortic valve (David I procedure) so to minimize the need for chronic anticoagulant therapy and antibiotic prophylaxis [109, 110]. While these surgical interventions can prevent further dilatation of the root and ascending aorta, careful monitoring is still required distal to the repair site to prevent dissection farther downstream [104]. Whenever possible, surgery in pediatric patients is delayed until adolescence so to avoid additional corrective interventions due to normal post-natal growth of the aorta [104].

6.5 Molecular Genetics

MFS is inherited as an autosomal dominant trait with an incidence of 1 per 5,000 live births of which ~25 % represent de novo mutations [76]. The *FBN1* gene resides on chromosome 15q21.1 and codes for a 350 kDa glycoprotein that mostly consists of 6-cysteines epidermal growth factor (EGF)-like and calcium-binding EGF (cb-EGF) modules interspersed by a few 8-cysteines motifs (TB/8-Cys) uniquely found in fibrillins and latent TGFβ-binding proteins (LTBPs) [5, 107]. Calcium binding stabilizes contiguous cb-EGFs into a rigid linear structure that is required for proper protein deposition in the ECM, polymerization into macromolecular aggregates, and protection from degradation by MMPs. *FBN1* defects include missense mutations and in-frame exon out-splicing or deletions that alter protein folding, secretion and/or assembly and enhance degradation of mutant molecules during tissue remodeling/repair, and mutations that cause nonsense-mediated RNA decay or whole gene deletion reducing the normal level of wild type proteins [38, 108]. Irrespective of whether *FBN1* mutations affect protein structure or expression, they similarly decrease the amount of immunoreactive fibrillin-1 in diseased tissues, indicating that the MFS phenotype largely reflects a sub-optimal threshold of functional microfibrils [109]. The vast majority of the more than a thousand MFS mutations identified thus far represent unique genetic lesions whose relative location and molecular identity are not predictors of phenotypic outcome [37]. Sole exception is the clustering of mutations causing the neonatal severe form of MFS within the middle third of fibrillin-1 where some mutations associated with adult MFS map as well [43, 110]. The possibility of deriving prognostic genotype-phenotype correlations is further complicated by the clinical variability of similar *FBN1* mutations among different patients or of the same mutation within individual families, which are both likely to reflect functional interactions between fibrillin-1 and other proteins (i.e., genetic modifiers).

While it is well established that MFS is a genetically homogenous condition, there are also rare instances in which *FBN1* mutations can cause clinically distinct phenotypes. Cases in point include the stiff skin syndrome (OMIM #184900), whose hallmark is severe dermal fibrosis associated with joint contracture and short stature, Weill-Marchesani syndrome (OMIM # 608328), which manifests MFS-like ocular manifestations but also exhibits short stature and increased joint stiffness and muscle mass, and acromicric dysplasia and geleophysic dysplasia (collectively referred to as acromilic dysplasias; OMIM #102370 and #614185, respectively), which overlap with Weill-Marchesani syndrome and to a lesser extent stiff skin syndrome, as they are characterized by severe short stature, joint stiffness and skin thickening [111–113]. These three conditions may therefore represent a clinical continuum resulting from different pathogenic mechanisms than those underlying MFS. The study of stiff skin syndrome has suggested that impaired interactions between extracellular fibrillin-1 assemblies and resident dermal cells represent one of such differentiating mechanisms [111]. This suggestion is based on the clustering of *FBN1* mutations in this extremely rare disorder around the sole integrin-binding site of fibrillin-1. Another distinctive mechanism emerging from recent findings in acromilic dysplasias is that interaction between fibrillin-1 and ADAMTSL2 might be required for proper assembly of an organized microfibfrilllar network, as the two ECM proteins bind to each other in vitro and some patients harbor *ADAMTSL2* mutations [113]. Shprintzen-Goldberg syndrome (OMIM #182212), which displays MFS-like skeletal and cardiovascular malformations as well as unique neurodevelopmental and cranial abnormalities, is another systemic disease of connective tissue very rarely associated with *FBN1* mutations [114].

6.6 Pathophysiology

Fibrillin-1 assemblies (microfibrils and elastic fibers) perform two critically important physiological functions; they provide the structural scaffold that imparts specific physical properties to various tissues and they regulate cell performance by interacting with integrin receptors and TGFβ family members. Microfibrils and elastic fibers are ubiquitous ECM components that are particularly abundant in tissues subject to stretching and expansile forces, in addition to being affected in MFS, such as the aortic wall, the perichondrium and the lens suspensory ligaments [115]. These correlative observations were originally interpreted to suggest that cardinal manifestations in MFS are accounted for loss of tissue integrity and implicitly key physical properties, such as aortic wall elasticity, physeal growth constrain, and ocular lens anchoring [9, 116]. Subsequent experiments with mice replicating the clinical spectrum of MFS have indicated that promiscuous TGFβ signaling (through both the canonical Smad2/3 [R-Smad] pathway and non-canonical mitogen-activated kinase [MAPK] pathways) is another major contributor to MFS pathogenesis [117–119]. Since these studies employed pan-TGFβ inhibitors, the identity of the TGFβ isoform(s) involved in various MFS manifestations remains to be determined.

Fibrillin-1 microfibrils localize, concentrate and stabilize latent TGFβ complexes by binding LTBPs [107]; they similarly interact in vitro with pro-BMP complexes conceivably to promote latency of bioactive ligands [120, 121]. It follows that *FBN1* mutations may also destabilize local growth factor bioavailability with negative consequences for resident cell performance [115]. Indeed, three mouse models of MFS have validated this hypothesis by implicating dysregulated TGFβ and BMP signaling in the pathogenesis of cardiovascular and skeletal manifestations. The emerging view from these animal studies is that *FBN1* mutations differently impact the formation, growth and function of various organ systems depending on how fibrillin-1 assemblies regulate the physical properties of individual tissues and the signaling of ECM-bound growth factors, and how different cell types respond to these highly contextual extrinsic stimuli [109].

Aortic aneurysm: Fbn1$^{mgR/mgR}$ mice produce ~20 % of the normal amount of fibrillin-1 and die from ruptured TAA within the first year of

life; they also exhibit severe rib cage and spine deformities, longer tubular bones, osteopenia, emphysema, DCM, and MVP [122, 123]. Newborn *Fbn1*$^{mgR/mgR}$ mice show a morphologically normal aortic wall that gradually degenerate through a maladaptive remodeling process that involves focal elastic lamellae calcification, inflammatory cell recruitment and activation, intimal hyperplasia, unorganized ECM accumulation and MMP-mediated elastolysis [122]. This mouse model of severely progressive MFS therefore demonstrated that secondary cellular events during post-natal life (as opposed to impaired elastogenesis during fetal development) account for aortic growth and degeneration. Newborn *Fbn1*$^{mgR/mgR}$ mice also exhibit impaired distal alveolar septation leading to destructive emphysema later in life, which is associated with increased TGFβ activity and epithelial cell death [119]. Perinatal TGFβ antagonism using a neutralizing antibody (Nab) has been shown to attenuate the severity of lung abnormalities, thereby linking fibrillin-1 deficiency and improper latent TGFβ activation to a specific MFS manifestation [119].

Fbn1$^{C1039G/+}$ mice express equal amounts of wild type and mutant fibrillin-1 molecules and have a normal life span because TAA does not precipitate vessel wall dissection and rupture [124]. *Fbn1*$^{C1039G/+}$ mice treated with either TGFβ Nab or the ARB losartan show a significant TAA improvement, as evidenced by normalized aortic wall thickness and architecture as well as TGFβ signaling [118], The last finding confirmed previous reports from animal models of cardiac and kidney fibrosis that the angiotensin type 1 receptor (AT1r) can stimulate TGFβ production through phosphorylation (p) of R-Smad proteins [125–127]. By contrast, the β-blocker propranolol, a drug commonly used to alleviate hemodynamic stress in MFS, has an intermediate benefit on aortic wall thickness but not on aortic wall architecture [118]. These mildly affected MFS mice therefore provided the experimental justification for launching several clinical trials to test whether or not ARB therapy is a more effective strategy than β-adrenergic receptor blockade against TAA progression in MFS patients [88, 89].

Based on the above studies, a model of aortic disease has been proposed whereby impaired sequestration of latent TGFβ complexes by a fibrillin-1 deficient matrix renders these signaling molecules more accessible to activation [119]. The model implies that the amount of available substrate (i.e.: latent TGFβ complexes) rather than substrate's activators (i.e.: MMPs, integrins and/or other molecules) is the limiting factor in aortic disease promotion. Subsequent work with *Fbn1*$^{C1039G/+}$ and *Fbn1*$^{-/-}$ mice (a.k.a. *Fbn1*$^{mgN/mgN}$ mice) has refined this disease model by implicating MAPKs in TAA formation [117, 128, 129]. In addition to high pR-Smad levels, the aortas of adult *Fbn1*$^{C1039G/+}$ mice also have greater than normal amounts of pERK1/2 that can be decreased by TGFβ Nab administration, implying growth factor signaling through both canonical and non-canonical pathways [130]. ERK1/2 activation was shown to be a major determinant of vascular disease as inhibition of this pathway improves TAA even more than TGFβ Nab without however normalizing pR-Smad levels [130]. Additional genetic and pharmacological findings indicated that signaling through AT1r drives ERK1/2 activation and that signaling through the angiotensin type 2 receptor (AT2r) inhibits it [129, 130]. Together, these observations suggested that fibrillin-1 mutations disrupt reciprocal interplays between angiotensin receptors and their downstream pathways that normally orchestrate aorta remodeling.

In spite of lacking fibrillin-1 molecules, *Fbn1*$^{-/-}$ mice complete fetal development and display a seemingly normal medial architecture at birth; however, they die soon after from catastrophic collapse of the aortic wall prior to overt expression of the secondary cellular abnormalities that characterize mouse models of adult MFS [128]. *Fbn1*$^{-/-}$ mice therefore recapitulate the early events that precipitate aortic disease in the neonatal lethal form of MFS. The aortas of newborn *Fbn1*$^{-/-}$ mice have abnormally high amounts of p-p38, a stress-response MAPK also involved in augmenting MMP activity [117]. Elevation of p-p38 MAPK levels in *Fbn1*$^{-/-}$ mice is detected prior to pR-Smad increase and can be partially reduced by p38 MAPK inhibition without however rescuing the

structural collapse of the aortic wall [117]. This mouse model of neonatal lethal MFS therefore suggested that, during the early stage of TAA formation, a mechanically impaired matrix stimulates MAPK-mediated stress responses promoting aortic tissue remodeling through ECM neo-synthesis (via TGFβ action) and degradation (via MMP action). This view furthermore predicts that dysregulated TGFβ bioavailability in the fibrillin-1 mutant matrix may exacerbate maladaptive tissue remodeling thereby leading to irreversible aortic wall degeneration [123].

As the aforementioned studies were performed with mice that replicate the neonatal lethal and mild (non-lethal) forms of MFS, the question arises whether the phenotypic outcome of anti-TGFβ therapy in these two animal models, albeit comparable, may reflect the targeting of the same disease effectors that however operate in distinct manners during the early (formation) and late (progression) stages of aortic disease. This question has important clinical implications given TGFβ central role in promoting physiological tissue maturation and growth during post-natal life, as well as ECM remodeling and repair in response to environmental stresses or injury [107]. In support of this argument, losartan treatment has been reported to normalize aortic diameter but not aortic wall architecture in *Fbn1*$^{mgR/mgR}$ mice, whose vascular severity is in between those of *Fbn1*$^{-/-}$ and *Fbn1*$^{C1039G/+}$ mice, with the result of delaying rather than preventing ruptured TAA [98, 123].

In conclusion, current evidence from genetic and pharmacological studies of MFS mice indicates that mutations in fibrillin-1 trigger multiple signaling (i.e., TGFβ, AT1r and AT2r pathways), cellular (i.e., SMC and immune cells) and catabolic events (i.e., MMP-mediated elastolysis) that cooperate in promoting and sustaining vascular disease onset and progression. A future research challenge to translate these findings into a more effective clinical management of aortic aneurysm in MFS is therefore to tease out the determinants responsible for disease onset from those supporting disease progression [131].

Osteopenia: Even though reduced BMD is a relatively minor problem in MFS, the study of osteopenia in *Fbn1* mutant mice has provided compelling evidence for organ-specific disease mechanisms. As such, these investigations have raised the possibility that therapeutic interventions in MFS should be tailored against individual manifestations. Increased bone resorption is the main cause of bone loss in adult (3 month-old) *Fbn1*$^{mgR/mgR}$ mice [123]. The phenotype is correlated at the cellular level with osteoblasts that differentiate more rapidly and support osteoclastogenesis more strongly than the wild type counterparts. As expected, osteopenia in MFS mice is normalized by systemic administration of alendronate, a bisphosphanate commonly used to prevent bone degradation.

Cultured osteoblasts from *Fbn1*$^{mgR/mgR}$ mice show elevated TGFβ and BMP signaling due to improper regulation of growth factor bioavailability by a fibrillin-1 deficient matrix. [123, 132] TGFβ and BMP complexes regulate bone formation differently. TGFβ signals promote osteoprogenitor cell recruitment from marrow stem cells (MSCs) and inhibit pre-osteoblast maturation, whereas BMP signals stimulate both processes [132, 133]. These growth factors are also involved in regulating bone resorption by stimulating osteoblasts to produce pro- and anti-osteoclastogenic factors [134]. Fibrillin-1 microfibrils therefore appear to act as extrinsic structural regulators of both bone anabolism and bone catabolism by calibrating the balance between local TGFβ and BMP signals. In line with this dual regulatory function, osteopenia is partially improved in *Fbn1*$^{mgR/mgR}$ mice treated with TGFβ Nab (our unpublished data). On the other hand, losartan administration does not improve BMD even though mutant osteoblasts express AT1r and AT1r signaling is unaffected [123]. Taken at face value, these findings exclude involvement in deregulated bone homeostasis of the same pathogenic mechanism responsible for TAA formation. Fibrillin-1 deficiency also impacts MSC performance, conceivably because of perturbed growth factors bioavailability, as bone marrow preparations from 3 month-old *Fbn1*$^{mgR/mgR}$ mice yield a greater number of colony-forming unit fibroblasts [135]. It follows that premature depletion of osteoprogenitor cells might exacerbate osteoclast-driven bone loss in

aging MFS mice, and that anti-TGFβ therapy alone might not counteract this disease process in the absence of additional interventions curbing abnormally high pro-osteogenic BMP signals.

Skeletal deformities are a significant morbidity factor in MFS, but the underlying mechanism remains a topic of speculations. One view argues that *FBN1* mutations lead to disproportionate bone lengthening because they impair the physical properties of the perichondrium, whereas another view postulates that dysregulated bioavailability of fibrillin-1 interacting growth factors is the principle driver of the abnormality [109, 136]. Our preliminary evidence from *Fbn1* conditional null mice suggests that the main tissue source of the phenotype is the perichondrium, which is involved in both imparting physeal constrain on bone lengthening and communicating with the growth plate during bone formation [137]. Future mouse studies are expected to delineate the physiological role of perichondrial fibrillin-1 and perhaps identify pharmacological means to curb post-natal bone overgrowth in MFS.

6.7 Future Perspectives

While improvements in medical and surgical therapy have nearly normalized life expectancy in MFS, there is an urgent need to properly evaluate the risks and benefits of current and emerging classes of medications so to formalize a new standard of care. As in the past, clinical progress will continue to rely on basic science findings and vice versa. Pre-clinical trials in mouse models of MFS will enable to compare the efficacy of different pharmacological formulations and multi-drug combinations. Application of computational systems biology protocols to the study of MFS will complement these investigations by providing an integrated and unbiased identification of new biological targets for therapy. Data from ongoing and future clinical trials in large and well-defined cohorts of MFS patients will in turn validate evidence gathered from mouse studies and in turn raise new questions that can be addressed experimentally. It is safe to anticipate that such an iterative process will ultimately lead to improved care of MFS patients, in addition to emphasizing the usefulness of characterizing relatively rare monogenic diseases to delineate fundamental pathophysiological mechanisms that are involved in more common clinical conditions.

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Loeys-Dietz Syndrome

7

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Abstract

Loeys-Dietz syndrome is an autosomal dominant aortic aneurysm syndrome characterized by multisystemic involvement. The most typical clinical triad includes hypertelorism, bifid uvula or cleft palate and aortic aneurysm with tortuosity. Natural history is significant for aortic dissection at smaller aortic diameter and arterial aneurysms throughout the arterial tree. The genetic cause is heterogeneous and includes mutations in genes encoding for components of the transforming growth factor beta (TGFβ) signalling pathway: *TGFBR1, TGFBR2, SMAD3* and *TGFB2*. Despite the loss of function nature of these mutations, the patient-derived aortic tissues show evidence of increased (rather than decreased) TGFβ signalling. These insights offer new options for therapeutic interventions.

Keywords

Loeys-Dietz syndrome • Hypertelorism • Aortic aneurysm with tortuosity • Aortic dissection • Mutations in *TGFBR1* • *TGFBR2* • *SMAD3* or *TGFB2* • Increased TGFβ signalling • Overlap with Marfan and Ehlers-Danlos syndrome

7.1 Introduction

The Loeys-Dietz syndrome (LDS, OMIM ID #609192) was first described by Loeys and Dietz in 2005. The initial paper presented ten probands with a novel aortic aneurysm syndrome characterized by the clinical triad of hypertelorism, bifid uvula/cleft palate and aortic/arterial aneurysms and tortuosity [1]. Although these presented the most typical characteristics, a widespread involvement of different organ systems was also recognized. These included craniofacial

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(e.g., craniosynostosis), skeletal (joint laxity and contractures), integumental (skin hyperextensibility, dural ectasia) and ocular findings (e.g., strabismus). Although LDS shows clinical overlap with Marfan syndrome (MFS), it can be clinically distinguished from the latter. Shared features include aortic root aneurysm, pectus deformities, scoliosis and arachnodactyly. Distinguishing findings are craniosynostosis, hypertelorism, cleft palate or bifid uvula, cervical spine instability, club feet, and most importantly widespread arterial aneurysms with tortuosity and early aortic rupture. Since the initial description of LDS, families with aortic aneurysms without significant outward features have also been described [2, 3].

7.2 Inheritance and Mutational Spectrum

LDS is an autosomal dominant disorder. About two third of cases are the consequence of *de novo* mutations, whereas the other one third are familial. In general, the more severe cases with marked craniofacial and skeletal findings are the consequence of a *de novo* mutation, whereas the milder cases tend to be familial. Both non-penetrance [4] and mosaicism [5] have been reported.

Two major genes have been initially associated with LDS. These genes encode the transforming growth factor β receptors 1 and 2, *TGFBR1* and *TGFBR2*. *TGFBR1* is located on chromosome 9q and contains 9 exons, whereas *TGFBR2* is positioned on chromosome 3p and contains 8 exons. Most recently, mutations in the gene encoding *SMAD3* have been associated with a condition called aneurysms osteoarthritis syndrome, showing a significant clinical overlap with LDS [6]. Finally, also mutations in *TGFB2* have been identified in patients with LDS-like presentations [7].

One third of the *TGFBR* mutations are identified in *TGFBR1* whereas the remainder is found in *TGFBR2* [4]. Mutations are primarily located in the serine-threonine kinase domain, the intracellular part of the TGFβ receptor. Although occasional nonsense mutations or small intragenic deletions have been described in *TGFBR2*, these were all predicted to escape nonsense-mediated-decay [4]. Deletions involving *TGFBR2* lead to an LDS-like phenotype but patients lack significant aortic disease [8]. Moreover, *TGFBR1* nonsense mutations or mutations predicted to cause a complete loss-of-function have been shown recently to lead to a skin cancer phenotype, multiple self-healing squamous epithelioma [9]. Haplo-insufficiency and loss-of-function were suggested as mutational mechanisms for both *SMAD3* and *TGFB2* mutations. All findings hitherto suggest that although the *TGFBR*-mutations in LDS are also predicted to lead to loss-of-function [10], some residual protein activity seems to be required to cause the LDS phenotype (see pathogenesis).

7.3 Signs and Symptoms

An overview of the clinical features of LDS is given in Table 7.1. LDS is characterized by four major groups of clinical findings, affecting the vascular, craniofacial, skeletal and cutaneous system [1]. Although some clinical overlap with MFS exists, highly prevalent distinguishing features in LDS are cleft palate/bifid uvula, hypertelorism and arterial tortuosity. Interestingly, in some patients the bifid uvula is the only visible marker to identify people at risk for aortic aneurysms.

7.3.1 Cardiovascular Manifestations

In the vascular system, the most common and prominent finding is the dilatation of the aortic root at the sinuses of Valsalva, which if undetected, leads to aortic dissection and rupture. These dissections have been described in patients as young as 6 months of age. Moreover, dissections have occurred at smaller diameters than those generally accepted at risk in MFS [11]. In addition to the aortic root aneurysms, aneurysms throughout the arterial tree have been described, most prominently in the side branches of the aorta and the cerebral circulation. Finally, another striking finding is the pres-

Table 7.1 Clinical features at initial diagnosis of LDS

Craniofacial features	
Craniosynostosis	15 %
Hypertelorism	48 %
Cleft palate/uvula	72 %
Exo/esotropia	18 %
Blue sclerae	23 %
Skeletal features	
Pectus deformity	51 %
Joint contractures	23 %
Joint hypermobility	50 %
Arachnodactyly	56 %
Club feet	22 %
Pes planus	51 %
Scoliosis	70 %
Cervical spine abnormality	39 %
Skin features	
Thin, translucent	33 %
Smooth, velvety	23 %
Easy bruising	24 %
Delayed wound healing	12 %
Herniae	25 %
Vascular findings	
Arterial tortuosity	92 %
Most common in head and neck vessels	
Carotids	55 %
Vertebrals	56 %
Intracranial	37 %
Ascending aorta	5 %
Aortic arch	10 %
Descending thoracic or	4 %
abdominal aorta, also other vessels (e.g. iliacs)	7 %
Aneurysms	
Aorta	
Root	87 %
Ascending	27 %
Arch	10 %
Desc thoracic	15 %
Abdominal	12 %
Vessel beyond Ao	30 %

ence of arterial tortuosity, which is usually most prominent in the head and neck vessels. Vertebral and carotid artery dissection and cerebral bleeding have been described; however, isolated carotid artery dissection in the absence of aortic root involvement has not been observed [1, 4, 12].

7.3.2 Skeletal Manifestations

Marfanoid skeletal features can be observed, although the actual overgrowth tends to be milder in LDS patients compared to MFS patients. Most typical LDS skeletal findings include pectus excavatum or pectus carinatum, scoliosis, joint laxity, arachnodactyly, talipes equinovarus and cervical spine malformation and/or instability. Arachnodactyly is present in some, but true dolichostenomelia (leading to an increase in the arm span-to-height ratio and a decrease in the upper-to-lower segment ratio) is less common in LDS than in MFS. The combined thumb and wrist signs are present in circa one-third of individuals with LDS. Joint hypermobility is very common and does include congenital hip dislocation and recurrent joint subluxations. Paradoxically, some individuals can show reduced joint mobility, especially of the hands (camptodactyly) and feet (club feet). Other recurrent skeletal findings include spondylolisthesis, acetabular protrusion and pes planus [1, 4]. Preliminary evidence suggests that individuals with LDS have an increased incidence of osteoporosis with increased fracture incidence and delayed bone healing [13].

7.3.3 (Cranio)facial manifestations

Most typical craniofacial features consist of ocular hypertelorism and the presence of a cleft palate, or its mildest presentation, a bifid uvula. Sometimes the uvula is not bifid but has an unusual broad appearance with or without a midline raphe. Another common presenting feature in the more severely affected patients is craniosynostosis. In the latter all sutures can be involved: most commonly the sagittal suture (resulting in dolichocephaly), but also the coronal suture (resulting in brachycephaly) and metopic suture (resulting in trigonocephaly). Other common craniofacial characteristics are malar flattening and retrognathia. Besides the hypertelorism, ocular manifestations include strabismus, blue sclerae and myopia, but the latter is less frequent and less severe than in MFS. Significant refractive errors can lead to amblyopia.

Retinal detachment has been reported rarely [1, 4]. In our experience, ectopia lentis is not observed, although in the literature minimal lens(sub)luxation has been reported [14]. Less common associated findings requiring further exploration include submandibular branchial cysts and defective tooth enamel [4].

7.3.4 Cutaneous Manifestations

In persons without craniofacial features, important cutaneous findings can provide the clue towards diagnosis. These skin findings show significant overlap with those observed in Ehlers-Danlos syndrome (EDS) and include velvety, thin, translucent skin, easy bruising (other than the lower legs) and dystrophic scars. Comparable to the vascular type of EDS, life-threatening complications, such as spontaneous bowel rupture and peripartal uterine rupture have been reported [4, 15]. Although in the past, type I and II LDS have been described based on the presence of these vascular EDS-like findings, we now believe these are the representation of a continuum within the LDS spectrum of disease.

7.3.5 Other Findings

Finally, a minority of affected individuals present developmental delay. When present, developmental delay is most often associated with craniosynostosis and/or hydrocephalus, suggesting that learning disability is an extremely rare primary manifestation of LDS. Common neuroradiological findings are dural ectasia and Arnold-Chiari type I malformation [16]. The precise incidence of those two findings is unknown.

Other recurrent findings that need further documentation include muscle hypoplasia, dental problems with enamel dysplasia, allergic disease with seasonal allergies, asthma/sinusitis, eczema and important gastro-intestinal problems: food allergy, eosinophilic esophagitis, inflammatory bowel disease.

7.4 Clinical Presentation of LDS-Related Diseases

Van de Laar et al. recently described another autosomal dominant variant of LDS, also called aneurysms osteoarthritis syndrome (AOS) [6]. AOS is characterized by aneurysms, dissections and tortuosity throughout the arterial tree in addition to craniofacial (including hypertelorism and abnormal palate/uvula), skeletal (including arachnodactyly and scoliosis) and cutaneous (including striae and velvety skin) symptoms and thus perfectly fits in the phenotypic spectrum of LDS. A distinguishing feature, however, might be the presence of early-onset osteoarthritis. In the initially published series, about 50 % of the patients present with osteochondritis dissecans and about 90 % of patients have vertebral disc degeneration, suggesting that these findings are very common in the *SMAD3* associated type of LDS [6]. Since the initial publication, however, it has become clear that not all *SMAD3* mutation positive patients do present with osteoarthritis [17, 18]. The cardiovascular severity of AOS seems similar to classical LDS with early onset dissections at smaller diameters and marked tortuosity [19, 20].

Finally, patients with mutations in the *TGFB2* gene, also present with an autosomal dominant disorder with many systemic features of both MFS and LDS. Features shared with MFS and LDS include aortic aneurysm, pectus deformity, arachnodactyly, scoliosis and skin striae. Features shared with LDS but not with MFS, consist of hypertelorism, bifid uvula, bicuspid aortic valve (BAV), arterial tortuosity, club feet and thin skin with easy bruising. Ectopia lentis was not observed [7].

Most recently, mutations in *SKI*, a functional repressor of TGFβ signaling, were identified as the cause of Shprintzen-Goldberg syndrome (SGS) [29]. SGS is characterized by craniosynostosis, distinctive craniofacial features with dolichocephaly, retrognathia, high arched palate, marfanoid skeletal changes including dolichostenomelia, arachnodactyly, camptodactyly, pes planus, pectus excavatum

Table 7.2 Differential diagnostic features of MFS, LDS and SGS

	MFS	LDS			SGS
Clinical feature	*FBN1*	*TGFBR1/TGFBR2*	*SMAD3*	*TGFB2*	*SKI*
Ectopia lentis	+++	–	–	–	–
Cleft palate/bifid uvula	–	++	+	+	+
Hypertelorism	–	++	+	+	++
Craniosynostosis	–	++	–	–	+++
Tall stature	+++	+	+	++	
Arachnodactyly	+++	++	+	+	++
Pectus deformity	++	++	++	++	++
Club foot	–	++	+	++	+
Osteoarthritis	+	+	+++	+	–
Aortic root aneurysm	+++	++	++	++	+
Arterial aneurysm	–	++	+	+	+
Arterial tortuosity	–	++	++	+	+
Early dissection	+	+++	++	+	–
Bicuspid aortic valve	–	++	+	+	+
Mitral valve insufficiency	++	+	+	++	+
Striae	++	+	+	+	+
Dural ectasia	+	+	+	+	+
Developmental delay	–	–	–	–	++

or carinatum, scoliosis, joint hypermobility, and contractures. Cardiovascular anomalies with mitral valve prolapse, mitral regurgitation, and aortic regurgitation may occur, but aortic root dilatation is usually mild. Minimal subcutaneous fat, abdominal wall defects, cryptorchidism in males, and myopia are also characteristic findings. Nearly all SGS patients present with developmental delay, a finding that is rare in LDS. Molecular analysis of a series of individuals with typical SGS did not reveal mutations in the *TGFBR1* or *TGFBR2* [1].

The major clinical findings of MFS, LDS and SGS are summarized in a comparative table (Table 7.2).

7.5 Diagnostic Criteria for LDS

Although no formal diagnostic criteria have been developed, *TGFBR1/2* genetic testing should be considered in the following scenarios:

1. Patients with the typical clinical triad of hypertelorism, cleft palate/bifid uvula and arterial tortuosity/aneurysm
2. Early onset aortic aneurysm with variable combination of other features including arachnodactyly, camptodactyly, club feet, craniosynostosis (all types), blue sclerae, thin skin with atrophic scars, easy bruising, joint hypermobility, BAV and patent ductus arteriosus (PDA), atrial and ventricular septum defects (ASD/VSD)
3. Patients with a MFS-like phenotype, especially those without ectopia lentis, but with aortic and skeletal features not fulfilling the MFS diagnostic criteria [21]
4. Families with autosomal dominant thoracic aortic aneurysms, especially those families with precocious aortic/arterial dissection, aortic disease beyond the aortic root (including cerebral arteries)
5. Patients with a clinical tableau reminiscent of vascular EDS (thin skin with atrophic scars, easy bruising, joint hypermobility) and normal type III collagen biochemistry
6. Isolated young probands with aortic root dilatation/dissection

If patients present with premature onset of osteoarthritis in addition to any of the above

clinical scenarios, *SMAD3* may be prioritized as the causal gene. If the clinical presentation is rather mild, mutation in *TGFB2* may also be considered. Although it should be stressed that the clinical overlap is so large, that it is impossible to predict the correct causal gene based on the clinical signs only. If craniosynostosis and intellectual disability are associated features, *SKI* might be the first gene to be analysed.

7.6 Differential Diagnosis

7.6.1 Syndromic Differential Diagnosis

7.6.1.1 Ehlers-Danlos Syndrome

EDS is a clinically and molecularly heterogeneous disorder [22]. Amongst the different subtypes, the vascular, valvular [23] and kyphoscoliosis type can present with significant cardiovascular complications.

The most typical clinical manifestations of vascular EDS include thin, translucent skin, characteristic facial appearance, vascular fragility demonstrated by extensive bruising and easy bleeding and spontaneous arterial/intestinal/uterine ruptures [22]. An abnormal type III collagen biochemistry confirms the diagnosis, but ultimate confirmation of the diagnosis lies in the identification of mutations in the *COL3A1* gene, encoding for the type III collagen α-chain 1. Interestingly, in a cohort of 40 patients displaying a vascular EDS-like phenotype but normal collagen III biochemistry, 30 % carried *TGFBR1/2* mutations [4], suggesting on the one hand that vascular EDS closely resembles LDS but on the other hand that *TGFBR* mutations may cause a broad spectrum of diseases associated with aortic aneurysms. Finally, arginine-to-cysteine mutations in *COL1A1* have been identified in a subset of affected individuals who typically present with aneurysms of the abdominal aorta and iliac arteries reminiscent of vascular EDS. For these cases, distinct abnormalities on collagen electrophoresis have been observed [24].

The valvular type of EDS is a rare form of EDS with early onset cardiac valvular dysfunction. This autosomal recessive condition is caused by nonsense mutations in *COL1A2*. Other recurrent findings include joint hypermobility and skin hyperextensibility [23].

Finally, aortic aneurysm and arterial rupture can also occur in the kyphoscoliotic form of EDS (the former type VI or Ocular-Scoliotic type). This generalized connective tissue disorder is characterized by kyphoscoliosis, joint laxity, muscle hypotonia, and, in some individuals, ocular problems. This autosomal recessive form of EDS is caused by deficient activity of the enzyme procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1, also called lysyl hydroxylase 1). The diagnosis of EDS, kyphoscoliotic type relies on the demonstration of an increased ratio of deoxypyridinoline to pyridinoline crosslinks in urine. Alternatively, an assay of lysyl hydroxylase enzyme activity in skin fibroblasts is diagnostic. Mutations in *PLOD1*, the gene encoding the enzyme lysyl hydroxylase 1, are causative [25].

7.6.1.2 Arterial Tortuosity Syndrome and Autosomal Recessive Cutis Laxa Type 1

Two other autosomal recessive connective tissue disorders present arterial tortuosity and aortic aneurysm as key findings.

The arterial tortuosity syndrome (ATS) is characterized by generalized tortuosity, elongation, stenosis and aneurysm formation in the major arteries. Patients often die at a young age due to cardiopulmonary complications. Features in common with LDS include arachnodactyly, hypertelorism, cleft palate and/or bifid uvula, joint laxity or contractions and micro/retrognathia. ATS is caused by loss-of-function mutations in *SLC2A10*, encoding GLUT10, which belongs to the glucose transporter family but its precise function remains unknown [26].

Autosomal recessive cutis laxa type 1 (ARCL1) is another connective tissue disorder characterized by vascular anomalies, lung emphysema and diverticulae of the urinary and gastrointestinal tract aside from the cutaneous symptoms. As in ATS, prognosis can be severely compromised by cardiopulmonary

complications. Mutations in two fibulin genes, *EFEMP2* (also called *FBLN4)* or *FBLN5*, are responsible for ARCL1 [27, 28]. Arterial aneurysms and tortuosity are very prominent in patients with *FBLN4* mutations, while the cutaneous manifestations in *FBLN4* patients are limited and vascular stenosis is more pronounced in *FBLN5* patients. As such, ARCL1 caused by *FBLN4* mutations can be categorized within the LDS spectrum.

7.6.2 Non-syndromic Differential Diagnosis

Non-syndromic types of thoracic aortic aneurysms and dissections (TAAD), or types in which only minor additional symptoms are present exist as well. Occasionally, mutations in *FBN1* [30] and in *TGFBR1/2* [2, 3] causing TAAD have been described, perhaps representing the mildest end of the MFS/LDS phenotypic spectrum. Up to now, three genes, coding for components of the vascular smooth muscle contractile apparatus have been associated with familial thoracic aortic aneurysm: *ACTA2*, coding for vascular smooth cell specific α-actin, *MYH11* (β-myosin heavy chain 11) and *MYLK* (myosin light chain kinase) [31–33]. *ACTA2* mutations have been identified in 14 % of TAAD patients [31], while *MYH11* mutations have been found in TAAD patients with persistent ductus arteriosus [33]. Additional symptoms that can be found in *ACTA2* mutation positive patients include persistent ductus arteriosus, bicuspid aortic valve, iris flocculi, cerebrovascular accidents, Moya-Moya disease and coronary artery disease [34]. Most recently, mutations in *MYLK* have been shown to account for a small subset of familial aortic aneurysmal disease [32].

7.7 Pathology

Histologic examination of aortic tissue from LDS patients reveals elastic fiber fragmentation, loss of elastin content, a marked excess of collagen and accumulation of amorphous matrix components in the aortic media. Electron miscroscopy shows loss of the intimate spatial association between elastin deposits and vascular smooth muscle cells [1]. These findings have been reported already in very young children undergoing early aortic surgery and do occur in the absence of inflammation, suggesting a severe defect in elastogenesis rather than secondary elastic fiber destruction. LDS aortic samples had significantly more diffuse medial degeneration compared with MFS and control samples, but the changes are not specific for LDS [1].

7.8 Biochemical Defects and Pathogenesis

For a long time, Marfan syndrome has been used as the sole paradigm for the pathogenetic study of thoracic aortic aneurysm. The study of Marfan mouse models has shifted our understanding of the pathogenetic mechanisms underlying this condition. In the past, it was believed that the structural deficiency of fibrillin-1 was responsible for many of the phenotypic characteristics, but recent work has also evoked a significant role for altered TGFβ signalling. It is now believed that deficient microfibrils fail to sequester TGFβ in an inactive state and that overactivation of the TGFβ signalling pathway contributes significantly to the disease pathogenesis. The discovery of the genetic basis of LDS, has deepened our insights into the role of TGFβ in aortic aneurysm formation.

LDS is most frequently caused by mutations in the genes encoding the TGFβ receptor subunits TβRI and TβRII. The majority of LDS mutations are missense mutations positioned within the intracellular kinase domain, impairing kinase activity but not altering receptor expression or trafficking [1, 4]. These mutations are predicted to cause loss-of-function of TβRI and TβRII. Interestingly, a recent report describes a cutaneous neoplastic phenotype without aortic or systemic involvement in people with heterozygous mutations that confer haplo-insufficiency for *TGFBR1* [9]. In LDS, it was hypothesized that loss-of-function of the TGFβ receptors could lead to a paradoxical upregulation of TGFβ

signalling. Indeed, aortic tissue-derived fibroblast studies documented that heterozygous patient cells show full preservation of the acute-phase response to TGFβ, and that patient-derived tissues show evidence of increased (rather than decreased) TGFβ signalling [1, 4]. While this finding intuitively corroborates the essential role of TGFβ in the pathogenesis of aortic aneurysm, it was not clear how a loss-of-function of the TGFβ receptors could lead to the same upregulation of TGFβ activity as seen in the Marfan mouse models.

The current data suggest that expression of a receptor with impaired kinase activity is necessary to generate the LDS phenotype and would be compatible with either a dominant-negative or complex gain-of-function mechanism of disease. On the one hand, at least two studies, either using heterozygous patient cells or co-transfection experiments, could not find evidence for dominant-negative activity [1, 14]. On the other hand, a third study provided a somewhat complicated argument for a dominant-negative mechanism despite evidence that co-transfection of equal amounts (both 1X) of DNA encoding wild-type and mutant receptor subunits did not result in less than half the signaling activity seen upon transfection of a 2X complement of wild-type DNA (as expected for a dominant-negative mechanism)[35]. Given that the TGFβ receptor complex involves association between two TβRI and two TβRII subunits, one might argue that dominant-negative activity is both intuitive and inevitable. However, recent evidence suggests that the individual TβRI:TβRII dimers within this tetrameric complex bind ligand and signal independently, yielding a dominant-negative mechanism untenable [36]. When considered in combination with the repetitive observation of paradoxically increased TGFβ activity in LDS patient tissues, hypotheses have focused on the prospect of excessive and nonproductive compensatory mechanisms, most likely proximately induced by an imbalance of the various signalling functions (e.g. canonical versus noncanonical) supported by TGFβ receptors in a given cell type or an imbalance of TGFβ signalling in general between distinct but neighbouring cell populations [37]. This hypothesis was first supported by accentuation of the aneurysm phenotype in *Fbn1*$^{C1039G/+}$ mice after the introduction of *Smad4* haploinsufficiency in the context of maintained high levels of Smad-dependent signalling and enhanced Smad-independent signalling [38]. Furthermore, loss-of-function mutations in *SMAD3* or *TGFB2* were also associated with an overall increased TGFβ signature in the aortic wall [6, 7].

Together, these findings indicate that TGFβ signalling is under the control of multiple feedback regulatory pathways. While adding to the complexity, the data support the contentions that many features of microfibril disorders likely manifest failure of proper regulation of TFGβ function, and that consideration of both primary and secondary events will be required to attain full mechanistic insight. Overall, the observations confirm the central role of TGFβ in the final common pathway leading to aortic aneurysms in different syndromes.

7.9 Treatment and Management

7.9.1 Natural History

Comparison of the natural history of Marfan syndrome and Loeys-Dietz syndrome has lead to two important lessons. First, in the most severe cases of LDS (with more outward features of LDS) aortic dissections at smaller diameters as in MFS have been observed, leading to the need for earlier surgical intervention (see below). Secondly, it has been observed that the aortic disease is far more widespread in LDS with aortic disease beyond the aortic root and prominent involvement of aortic sidebranches, necessitating a complete imaging of the arterial tree from head to pelvis.

7.9.2 Medical Treatment

Many of the treatment strategies in LDS are derived from knowledge derived from MFS patient management. The current treatment for

aortic aneurysms in MFS is not causal and purely symptomatic. Preventive treatment with beta-blockers is believed to slow down the aortic root growth but in general this does not prevent aortic surgery at later age. Based on initial experiments that demonstrated the rescue of the lung phenotype in Marfan mouse models through the use of TGFβ neutralizing antibodies [39], it was hypothesized that similar treatments may be beneficial for the aortic phenotype in MFS patients. Proof-of-principle was obtained from a Marfan mouse trial [40]. The intraperitoneal injection of TGFβ neutralizing antibody blocked aortic root growth in these mice. Subsequently, similar results were obtained using an angiotensin II type 1 receptor blocker, losartan. Losartan does not only have an effect on the renin-angiotensin-aldosterone axis but has also an effect on TGFβ signaling. It is believed to reduce both the total and active amount of TGFβ in the extracellular matrix, probably through effects on thrombospondin, a TGFβ activator. In a placebo-controlled trial on Marfan mice, losartan resulted in significantly reduced aortic growth compared to atenolol, despite the similar hemodynamic effect. In addition to a major effect on the aortic growth, the histology of elastic fibers in the aortic wall of the losartan treated MFS mice was also indistinguishable from wild type mice [40].

The beneficial effect of angiotensin receptor blocker treatment on aortic growth was confirmed in a preliminary observational study in severely affected pediatric MFS patients. Similar to the MFS mice, a significant decrease in rate of change of aortic root dimension after starting angiotensin receptor blocker therapy was observed. Again, as there was no difference in the effect of hemodynamic parameters, the data suggest that these achieved protective effects were likely to be attributed to TGFβ antagonism [41]. This study has provided the first evidence for a significant benefit of angiotensin receptor blocking agents over current therapies in reducing aortic root dilation in severe pediatric MFS patients.

Based on the mouse data and the preliminary human study a large, randomized clinical trial in MFS patients has been initiated. This trial, supported by the Pediatric Heart Network through the U.S. National Heart, Lung and Blood Institute (NHLBI), compares atenolol with losartan treatment in more than 600 patients for a 3-year treatment [42]. In addition, a dozen other trials with different designs and inclusion criteria have been initiated in Belgium, France, Italy, The Netherlands, Taiwan and the United Kingdom [43–46]. As some of these studies might be underpowered, it is anticipated that a meta-analysis of trials with similar design will be necessary [47].

7.9.3 Surgical Treatment

Given the safety and the increasing availability of the valve-sparing procedure, the following recommendations have been issued for aortic surgery in LDS [48]. First, for young children with severe systemic findings of LDS, surgical repair of the ascending aorta should be considered once the maximal dimension exceeds three standard deviations and the aortic annulus exceeds 1.8 cm, allowing the placement of a graft of sufficient size to accommodate growth. Second, for adolescents and adults, surgical repair of the ascending aorta should be considered once the maximal dimension approaches 4.0 cm. This advice is based on both numerous examples of documented aortic dissection in adults with aortic root dimensions at or below 4.0 cm and the excellent outcome of prophylactic surgery. An extensive family history of larger aortic dimension without dissection could alter this practice for individual patients [49].

7.10 Genetic Counseling

LDS is inherited in an autosomal dominant manner. About one quarter of LDS patients has an affected parent whereas approximately three quarters of probands have LDS as the result of a *de novo* mutation. If the parent is affected, each child has a 50 % chance of inheriting the mutation and thus the disorder. Prenatal diagnosis for pregnancies at increased risk for LDS is possible if the disease-causing mutation in the family is known.

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8 Connective Tissue Disorders and Cardiovascular Complications: The Indomitable Role of Transforming Growth Factor-Beta Signaling

Jason B. Wheeler, John S. Ikonomidis, and Jeffrey A. Jones

Abstract

Marfan Syndrome (MFS) and Loeys-Dietz Syndrome (LDS) represent heritable connective tissue disorders that cosegregate with a similar pattern of cardiovascular defects (thoracic aortic aneurysm, mitral valve prolapse/regurgitation, and aortic root dilatation with regurgitation). This pattern of cardiovascular defects appears to be expressed along a spectrum of severity in many heritable connective tissue disorders and raises suspicion of a relationship between the normal development of connective tissues and the cardiovascular system. Given the evidence of increased transforming growth factor-beta (TGF-β) signaling in MFS and LDS, this signaling pathway may represent the common link in this relationship. To further explore this hypothetical link, this chapter will review the TGF-β signaling pathway, heritable connective tissue syndromes related to TGF-β receptor (TGFBR) mutations, and discuss the pathogenic contribution of TGF-β to these syndromes with a primary focus on the cardiovascular system.

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Shprintzen-Goldberg syndrome, hereditary hemorrhagic telangiectasia (HHT) • Marfan syndrome (MFS) • Loeys-Dietz syndrome (LDS) • Primary pulmonary hypertension • Fibrodysplasia ossificans progressiva (FOP) • Familial thoracic aortic aneurysm and dissection syndrome (FTAAD) • Smad • TGF-β receptor • Curacao diagnostic criteria

Abbreviations

AAT	Aortic Aneurysm Thoracic
ACE	Angiotensin Converting Enzyme
ALK-1	Activin Receptor-like Kinase 1
ALK-3	Activin Receptor-like Kinase 3
ALK-5	Activin Receptor-like Kinase 5
AOS	Aneurysm-Osteoarthritis Syndrome
ATI	Angiotensin II Receptor Type I
ATII	Angiotensin II Receptor Type II
ATS	Arterial Tortuosity Syndrome
AVM	Arteriovenous Malformation
BAV	Bicuspid Aortic Valve
BMP	Bone Morphogenetic Protein
BMPR1A	Bone Morphogenetic Protein Receptor 1A
BMPR2	Bone Morphogenetic Protein Receptor 2
Co-Smad	Common Smad
CTGF	Connective Tissue Growth Factor
ECM	Extracellular Matrix
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
EVAR	Endovascular Aortic Repair
FBN1	Fibrillin-1
FOP	Fibrodysplasia Ossificans Progressiva
FTAAD	Familial Thoracic Aortic Aneurysm and Dissection Syndrome
GI	Gastrointestinal
HHT1	Hereditary Hemorrhagic Telangiectasia, Type 1
HHT2	Hereditary Hemorrhagic Telangiectasia, Type 2
JNK	c-Jun N-terminal Kinase
LAP	Latent Associated Protein
LDS	Loeys-Dietz Syndrome
LLC	Large Latent Complex
LTBP	Latent Transforming Growth Factor-Beta Binding Protein
MAPK	Mitogen-Activated Protein Kinase
MFS	Marfan Syndrome
MMPs	Matrix Metalloproteinases
MVP	Mitral Valve Prolapse
OMIM	Online Mendelian Inheritance in Man
PAH	Pulmonary Artery Hypertension
PDA	Patent Ductus Arteriosus
PI3K	Phosphoinositide 3-Kinase
RA	Rheumatoid Arthritis
R-Smad	Receptor Smad
SARA	Smad Anchor for Receptor Activation
SGS	Shprintzen-Goldberg Syndrome
SLC2A10	Solute Carrier Family 2, Facilitated Glucose Transporter Member 10
SLE	Systemic Lupus Erythematosus
Smurf	Smad Ubiquitination Regulatory Factor
TAA	Thoracic Aortic Aneurysm
TAK1	Transforming Growth Factor-Beta Associated Kinase 1
TFs	Transcription Factors
TGF-β	Transforming Growth Factor-Beta
TGFBRI	Transforming Growth Factor-Beta Receptor, Type-I
TGFBRII	Transforming Growth Factor-Beta Receptor, Type-II
TIMPs	Tissue Inhibitors of Matrix Metalloproteinases
TRAF6	Tumor Necrosis Factor Receptor Associated Factor 6

8.1 Introduction

Marfan syndrome (MFS) is a well described connective tissue disorder characterized by musculoskeletal, ocular, and cardiovascular defects including: ascending aortic aneurysm with dissection, mitral valve prolapse (MVP)/regurgitation,

and aortic root dilatation with regurgitation [1] and it is discussed to considerable detail in Chap. 6 by Cook and Ramirez. A mutation in fibrillin-1 (FBN1), a protein component of microfibrils, accounts for more than 90 % of MFS [2]. Fibrillin-1 was demonstrated through multiple studies to interact with and sequester latent transforming growth factor-beta (TGF-β) within the extracellular matrix (ECM) [3–6]. In 2003, Neptune et al. hypothesized that the loss of microfibrils may have an effect on the sequestration of TGF-β within the ECM and demonstrated that TGF-β signaling was markedly activated within lung tissue of a mouse MFS model [7]. Furthermore, the emphysematous lung phenotype of the MFS mice was restored to wild type with anti-TGF-β antibody, strongly suggesting that TGF-β signaling dysregulation contributed to the pathogenesis of MFS [7].

Subsequently in 2005, Loeys and Dietz described a cohort of patients with a connective tissue disorder that significantly overlapped with the phenotype of MFS [8] (see also Chap. 7). Both disorders exhibit a marfanoid habitus (pectus deformity, arachnodactyly-elongated fingers, scoliosis, and dolichostenomelia-elongated limbs), valvular prolapse/regurgitation, and an arterial aneurysm with dissection phenotype [8]. Additionally, Loeys and Dietz identified mutations within type-I (TGFBRI) or II (TGFBRII) TGF-β receptors in these patients [8]. Interestingly, despite mutated receptors incapable of propagating signal, patients with Loeys-Dietz syndrome (LDS) paradoxically exhibited indications of increased TGF-β signaling: increased expression of collagen and connective tissue growth factor (CTGF), much like MFS patients [8].

Taken together, MFS and LDS represent connective tissue disorders that cosegregate with a similar pattern of cardiovascular defects. This pattern of cardiovascular defects appears to be expressed along a spectrum of severity in many heritable connective tissue disorders and raises suspicion of a relationship between the normal development of connective tissues and the cardiovascular system. Given the evidence of increased TGF-β signaling in MFS and LDS, this signaling pathway may represent the common link in this relationship. To further explore this hypothetical link, this chapter will review the TGF-β signaling pathway, heritable connective tissue syndromes related to TGF-β signaling-particularly TGFBR mutations, and discuss the pathogenic contribution of TGF-β to these syndromes with a primary focus on the cardiovascular system.

8.2 TGF-β, Signaling Pathways, and Physiological Effects

Transforming growth factor-β is a soluble cytokine secreted by cells in the form of a large latent complex (LLC) composed of a homodimer of mature TGF-β peptide, a homodimer of TGF-β's inactive cleaved peptide fragment (latent associated protein, LAP), and latent transforming growth factor binding protein (LTBP) [9]. Motifs within fibrillin-1 interact with LTBP and target the LLC to the ECM [6]. Thus, the ECM serves to sequester and concentrate TGF-β in locations where it may be rapidly activated when needed [10]. Indeed, the ECM is no longer thought to be a passive structural support but rather a dynamic regulator of growth factor bioavailability and signaling [11, 12].

8.2.1 "Canonical" TGF-β Signaling Pathway

Mature TGF-β (types 1–3) is activated by release from the LAP through multiple mechanisms including: direct proteolysis, by non-proteolytic dissociation mediated by thrombospondin-1 or integrin $\alpha_v\beta_6$, as well as exposure to reactive oxygen species, or low pH [13]. Once activated, TGF-β is free to bind a TGF-β receptor in the first step of the signaling cascade [9]. Transforming growth factor-β receptors have been subdivided into three types. Type-I (also known as Activin receptor-like kinase 5/ALK-5 or TGFBRI) and type-II are the primary receptors of the classical - or "canonical"-pathway and both possess serine/threonine kinase activity. Type-III (also known as betaglycan) is an accessory receptor that binds TGF-β and presents it to the type-I and II receptor complex [9]. Other receptors that bind and signal in response to TGF-β include endoglin

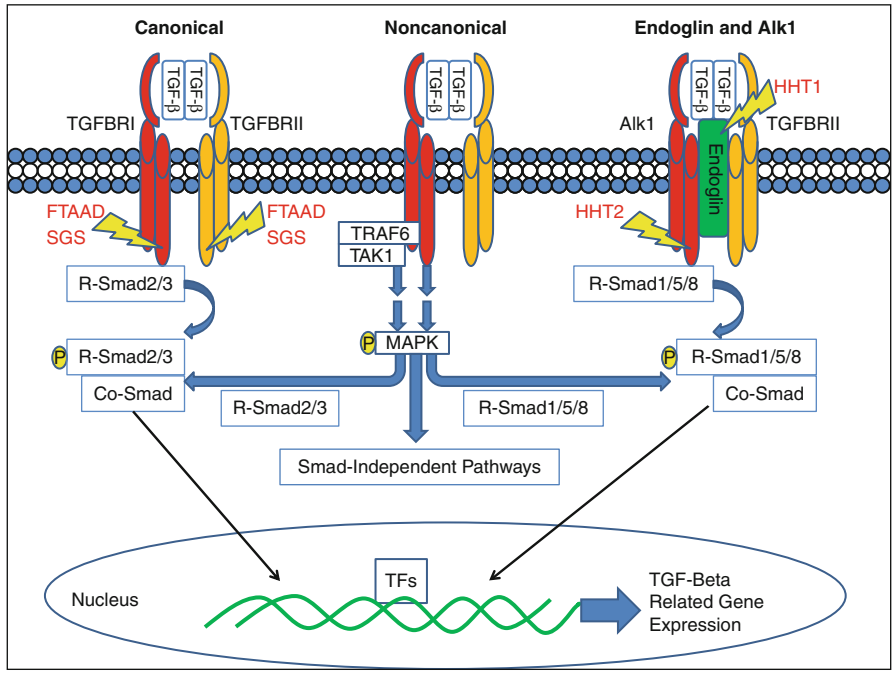

Fig. 8.1 Canonical, Noncanonical, and Endoglin/ALK-1 signaling pathways. Both Canonical and Endoglin/ALK-1 TGF-Beta signaling is mediated by the phosphorylation of distinct receptor Smad proteins. Nuclear translocation requires Co-Smad binding in both pathways. Inside the nucleus, R- and Co-Smads form a complex with transcription factors (TFs) to either repress or activate TGF-Beta related gene expression. The non-canonical pathway is mediated by TGFBRI, TRAF6, and TAK1 and results in the phosphorylation of MAPKs such as Erk1/2, JNK, and p38. These MAPKs can re-enter the Smad-dependent pathway through phosphorylation or mediate downstream signaling through other Smad-independent pathways. The lightning bolts represent mutations to the indicated proteins causative of the syndromes listed in *red*

(type-III receptor) and the Activin receptor-like kinase 1(ALK-1), a type-I receptor family member.

After release from the ECM, mature TGF-β first binds a homodimer of the type-II receptor inducing an autophosphorylation event. This, in turn, recruits a homodimer of the type-I receptor forming the complete ligand-receptor complex. The type-II receptor then activates the type-I receptor via transphosphorylation [14]. The kinase domain of the activated type-I receptor propagates the intracellular signal through the phosphorylation of specific receptor-regulated Smad proteins (R-Smads; Smad 1, 2, 3, 5, and 8), which are the second messengers of the canonical TGF-β signaling pathway. For example, activation of the type-I receptor TGFBRI, results in the phosphorylation of Smad 2 or 3; while activation of the type-I receptor ALK-1 results in the phosphorylation of Smad 1, 5 or 8. The choice of Smad is likely tissue-specific and context-dependent. The phosphorylated R-Smad then interacts with a common Smad or "co-Smad" (Smad4), which induces translocation of the complex to the nucleus. The nuclear Smad complex along with multiple co-regulatory factors form a transcription regulating complex capable of activating or repressing TGF-β associated genes [15, 16] (Fig. 8.1).

Activation of the TGF-β system stimulates a number of diverse cellular processes, such as cell growth, proliferation and apoptosis and therefore requires strict regulation at multiple levels. An example of this regulation, is the negative feedback of inhibitory Smads (I-Smads; Smad6 and 7) induced by TGF-β stimulation [17]. Smad6 exerts its effects by binding directly to type-I receptors and blunting R-Smad phosphorylation [18].

Smad6 also inhibits signaling by competing with Smad4 for receptor Smad binding sites, reducing nuclear translocation [19]. Smad7 inhibits TGF-β signaling by targeting TGFBRI and II for ubiquitination and subsequent degradation, through the recruitment of Smurfs 1 and 2 (Smad ubiquitination regulatory factor 1 and 2) [20–22]. Additionally, many regulatory proteins influence the bioavailability of TGF- β, such as the structurally related scavenging proteoglycans decorin and biglycan, which bind and reduce its availability for signaling [23–25].

8.2.2 Alternate "Noncanonical" TGF-β Signaling Pathways

Recent studies have expanded upon the hypothesis that TGF-β signaling can occur independently of Smad mediators, through alternative pathways. Several alternative signaling pathways exist: (1) type-I receptors signaling in the absence of Smads [26–30]; (2) type-II receptors signaling without type-I receptors [31, 32]; (3) R-Smad signaling to parallel pathways [33–35]; and (4) activation of R-Smads independent of TGFBRs [36, 37]. However, downstream intracellular mediators of these alternative pathways are not as well understood as the Smad proteins. Studies of noncanonical signaling in FBN1 deficient mice have proved helpful in this regard. Carta et al. demonstrated in vivo that p38 mitogen-activated protein kinase (p38 MAPK) mediated phosphorylation of Smad2/3, which was attenuated with p38 MAPK inhibitors suggesting independence from TGFBRI [38]. Further studies performed by independent groups elucidated the TGF-β dependent activation of p38 MAPK independently of Smad proteins. Tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, was demonstrated to associate with TGFBRI in a TGF-β dependent manner [39, 40]. This newly formed complex recruits and activates TGF-β associated kinase 1 (TAK1), which activates p38 MAPK via phosphorylation [39, 40] (Fig. 8.1). In addition to p38 MAPK, TGF-β can activate many other signal pathways not directly involving Smads: extracellular-signal regulated kinase 1 and 2 (ERK1/2) [41], c-Jun N-terminal kinase (JNK) [39, 40], and phosphoinositide 3-kinase-Akt (PI3K-Akt) [42]. Importantly, the canonical and noncanonical pathways appear to exert differential effects on the connective tissues within the ECM.

Transforming growth factor-β signaling is known to contribute to a number of disparate and opposing physiologic processes including angiogenesis, proliferation, differentiation, apoptosis, and wound healing, and is an established modulator of ECM structure and composition [43, 44]. Within the vascular ECM, TGF-β demonstrates opposing effects by its involvement in both matrix deposition and degradation. Recent evidence suggests that stimulation of a particular pathway determines whether deposition or degradation will predominate. Stimulation via the canonical pathway induces profibrotic effects including increased ECM protein deposition (collagen and elastin) [45], decreased expression of proteolytic enzymes (matrix metalloproteinases, MMPs) [46], and increased proteolytic inhibition (tissue inhibitors of MMPs, TIMPs) [47]. Alternatively, stimulation via the noncanonical pathway degrades matrix proteins through increased proteolysis via MMPs (2, 9, and 13) [48] and increased MMP activation via plasminogen activators [49]. Thus, matrix degradation appears to occur through Smad-independent pathways, though Smad activation may also be involved [50]. Noncanonical p38 MAPK mediated signaling has been associated with MMP-2 and -9 production and release in breast cancer cells [48]. In vitro expression of MMP-13 by rat osteoblasts was dependent on p38 MAPK, Smad2 (classical pathway), and extracellular signal-regulated kinase (ERK) 1/2 signaling [51]. This dual regulation underscores the importance of TGF-β as a key regulatory factor in maintaining homeostatic balance within the structure and composition of the ECM and implicates the dysregulation of the noncanonical signaling pathways in disorders associated with elevated TGF-β signaling and ECM degradation.

It must be recognized that the TGF-β family of ligands is part of a larger "superfamily" of growth factors and receptors, which includes bone morphogenetic proteins (BMPs), activins, and inhibins [16]. These superfamily members

also occupy roles in normal connective tissue development and repair. Like the TGF-β family, dysregulation of BMP signaling has been implicated in heritable connective tissue disorders. Mutations within activin receptor-like kinase 2, a type-I BMP receptor, are associated with fibrodysplasia ossificans progressive (FOP), a skeletal dysplasia characterized by progressive heterotopic bone formation [52]. Activin and inhibin signaling dysregulation has not been linked to heritable connective tissue disorders. However, activin signaling has been implicated in the regulation of wound healing and scar formation, processes dependent upon normal connective tissue remodeling [53]. While the TGF-β family is an established modulator of ECM remodeling, it may also occupy a similar role in vascular development. Evidence for this may be seen in the range of vascular abnormalities characteristic of TGFBR-mutation-related connective tissue disorders.

8.3 TGF-β Receptor Related Connective Tissue Disorders

To date several heritable connective tissue disorders have been associated with mutations in TGFBRs (and therefore disturbances in TGF-β signaling) including LDS, familial thoracic aortic aneurysm and dissection syndrome (FTAAD), Shprintzen-Goldberg syndrome (SGS), and hereditary hemorrhagic telangiectasia (HHT) (Table 8.1). Interestingly, each of these disorders also display unique cardiovascular manifestations, resulting in a spectrum of disorders ranging from heart valve defects to thoracic aortic aneurysms, characteristic of MFS and LDS, the prototypical disorders of aberrant enhanced TGF-β signaling. These disorders have been placed in context below.

8.3.1 Familial Thoracic Aortic Aneurysm and Dissection Syndrome (FTAAD)

Marfan syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos (type-IV) syndrome are the primary inherited connective tissue disorders associated with thoracic aortic aneurysms (TAA). However, many patients presenting with a history of familial TAAs and dissections cannot be classified into any of these syndromes. These TAAs and dissections have a heterogeneous etiology, and at least 7 associated gene mutations have been identified in the Online Mendelian Inheritance in Man (OMIM) database: Aortic Aneurysm Thoracic (AAT) 1–7. Two of these mutations, AAT5 (OMIM #610380) and AAT3 (OMIM #608967) are located within the genes for TGFBRI and TGFBRII, respectively [54]. As with most aneurysm patients, the initial presentation event is often incidentally discovered as aortic dilatation, dissection or sudden death [55–57]. Subsequently, an aortopathy syndrome is suspected when a family history of early aortic disease or sudden death is revealed or the constellation of unique connective tissue symptoms (marfanoid habitus like MFS and LDS and/or the FTAAD specific iris flocculi) provokes suspicion. While these seemingly non-syndromic TAAs and dissections may be the result of contributing risk factors (e.g., hypertension, atherosclerosis), it has been reported that almost 20 % of these patients have a first degree relative with a similar presentation, suggesting a genetic predisposition [58]. Diagnosing this group of patients is complicated due to the autosomal dominant inheritance with variable penetrance and expression [59]. Accordingly, these cases have been grouped as familial thoracic aortic aneurysm and dissection syndrome (FTAAD) and encompass all familial cases not captured by defined syndromes.

Several vascular and cardiac pathologies have been associated with FTAAD, signs and symptoms of which may be detected during a physical exam. The primary vascular disturbance in FTAAD involves aneurysmal dilatation (>1.5× the normal diameter) and dissection (an intimal tear that initiates progressive medial separation) of the thoracic aorta, most commonly in the ascending aorta [60]. Symptoms of aortic rupture and dissection include tearing chest pain, hypotension, differential pulse pressures, and rapid clinical decompensation [61]. Like LDS, FTAAD patients can also present with abdominal aortic aneurysm or cerebral aneurysms [54]. Arterial tortuosity (twisted, corkscrew arteries), which is

Table 8.1 TGF-β related heritable connective tissue disorders

Connective tissue syndrome	Associated mutations	Connective tissue manifestations	Cardiovascular manifestations	References
Shprintzen-Goldberg syndrome (SGS)	Reported FBN1, TGFBR I & II OMIM#182212 (FBN1)	Marfanoid habitus: dolichostenomelia, reduced upper:lower body ratio, scoliosis, pectus excavatum or carinatum; club foot; flat foot; hernias; scaphocephaly; craniosynostosis; digital contractures; Chiari-I; osteopenia	Mitral valve prolapse and regurgitation; aortic valve insufficiency; occasional aortic root dilatation	Greally et al. 1998 [75], Van Steensel et al. 2008 [146]
Loeys-Dietz syndrome (LDS)	TGFBRI & II OMIM#609192	Bifid uvula; cleft palate; clubfoot; hypertelorism; thin/velvety skin; blue sclera; cervical anomaly/instability; craniosynostosis; scoliosis; dural ectasia; protrusion acetabuli; lax joints	Ascending aortic aneurysm and dissection; diffuse arterial tortuosity and aneurysms; easy bruising; mitral valve prolapse and regurgitation	Loeys et al. 2005, 2006 [8, 59]
Familial thoracic aortic aneurysm and dissection syndrome (FTAAD)	AAT1-7 AAT5 TGFBRI OMIM#610380 AAT3 TGFBRII OMIM#608967	Marfanoid Habitus: dolichostenomelia, reduced upper:lower body ratio, scoliosis, pectus excavatum or carinatum; livedo reticularis; iris flocculi	Ascending and aortic root aneurysm and dissection; mitral valve prolapse and regurgitation	Gleason, TG. 2005 [155], Pannu et al. 2005 [156]
Marfan syndrome (MFS)	FBN1 OMIM#154700	Marfanoid habitus: dolichostenomelia, reduced upper:lower body ratio, scoliosis, pectus excavatum or carinatum; protrusio acetabuli; ectopia lentis; high arched palate; dural ectasia; lax joints	Ascending aortic aneurysm involving sinuses of Valsalva and dissection; aortic root dilatation with possible valve insufficiency; mitral valve prolapse and regurgitation	Judge et al. 2005 [1], Dietz et al. 1993 [2]
Aneurysm-osteoarthritis syndrome (AOS)	Smad3	Early onset osteoarthritis; osteochondritis dissecans; mild hypertelorism; abnormal uvula	Aortic aneurysms and dissection; tortuosity of large and medium sized vessels, even intracranial	Van de Laar et al. 2011, 2012 [71, 158]
Arterial tortuosity syndrome (ATS)	SLC2A10 OMIM#208050	High palate; skin and joint laxity; hernias; keratoconus; facies; micrognathia; contractures; arachnodactyly	Large and medium vessel tortuosity; diffuse aneurysms; aortic regurgitation; telangiectases; pulmonary artery stenoses and aneurysms	Callewaert et al. 2008 [157], Coucke et al. 2006 [73]
Hereditary hemorrhagic telangiectasia (HHT)	HHT1 TGFBR Type-III (Endoglin; ENG) OMIM#187300 HHT2 TGFBR Type-I (Activin receptor-like kinase-1/ALK-1) OMIM#600376	Specialized connective tissues: blood-thrombophilia; lymphatic tissue-immunodeficiency	Diffuse GI and mucocutaneous telangiectasais; arteriovenous malformations in lungs, brain and liver; nosebleeds; easy bleeding and bruising; iron deficiency anemia; pulmonary artery hypertension	Govani et al. 2009 [97], Fernandez et al. 2006 [154]

also a key feature of LDS, may also be seen in medium to large arteries (e.g. carotid arteries, aorta, etc.) [54]. Interestingly, some FTAAD patients exhibit Moyamoya disease, characterized by occlusive intimal thickening of the primary cerebral vessels progressing to transient ischemic events and stroke [62, 63].

Cardiac manifestations of FTAAD include patent ductus arteriosus (PDA), aneurysm of the aortic root, and bicuspid aortic valve (BAV) [64]. Bicuspid aortic valves occur in 1–2 % of the population and are the most common cardiac malformation. With age, BAVs calcify prematurely and may result in aortic stenosis or regurgitation, both of which produce distinctive murmurs and progressive symptoms of heart failure [64]. A PDA produces a continuous murmur and with time increased pulmonary blood flow will induce pulmonary hypertension [65].

8.3.1.1 Paradoxical Signaling by Mutated TGFBRs in FTAAD

Thoracic aortic aneurysms are characterized by progressive ECM degradation, elastin fragmentation, smooth muscle apoptosis and dilatation with or without dissection, likely due to an imbalance between matrix production and proteolysis [66]. The balance between ECM deposition and degradation is tightly regulated, and the mechanism of the loss of balance in TAAs is not clearly understood. For example, recent evidence uncovered paradoxical effects in the TGF-β signaling pathway, specifically identifying mutations in TGFBRs that resulted in elevated TGF-β signaling [8, 67]. Several mechanisms, however, have been proposed.

First, that heterozygous mutation of TGFBRs may enhance TGF-β signaling in functional TGFBR complexes by facilitating ligand interaction much like the role of type-III TGFBRs (endoglin and betaglycan). This was hypothesized after authors of a study using transgenic mice with a fibroblast-specific heterozygous TGFBRII mutation noticed increased pulmonary and dermal collagen deposition rather than a dominant negative phenotype which was expected to reduce deposition [67, 68]. Second, alternate pathways of receptor recycling could also explain the enhanced signaling seen. Internalization of TGFBRs is mediated by either clathrin- or caveolin- dependent endocytosis [69]. A plasma membrane protein, Smad anchor for receptor activation (SARA) binds TGFBRII and mediates interactions with Smad2 and the clathrin endocytosis pathway leading to receptor recycling [69]. A mutation that enhanced interaction of TGFBRII with SARA could increase signaling and favor recycling over degradation. Alternatively, the Smurfs (Smad ubiquitination regulatory factors) mediate TGF-β receptor interactions with the caveolin pathway [69, 70]. The interaction of the TGFBR complex with Smad7 recruits Smurf1 and 2, activating the caveolin pathway and leading to proteasomal degradation of the receptors. In this case, a TGFBR mutation that decreased interaction with either Smad7 or the Smurf proteins could decrease receptor degradation and prolong signaling. Finally, mutated TGFBRs may enhance signaling because their ability to form functional signaling platforms is unaffected by mutation. These signaling platforms are sites where multiple signaling intermediates aggregate. This was demonstrated when Smad3 was found to be phosphorylated by PI3K at residues not within TGFBRI's target site after TGF-β stimulation and increased collagen expression [36]. This suggests that those mutated TGFBRs with intact TGF-β binding but deficient kinase domains may still induce TGF-β dependent signaling through alternate pathways. Thus, enhanced TGF-β signaling may drive vascular ECM destruction through non-Smad signaling pathways alone, as a result of imbalanced homeostasis having direct implications for the formation and progression of thoracic aortic aneurysms and dissections.

Lending support to the pivotal role of TGF-β in connective tissues and the cardiovascular system, heritable mutations in downstream and upstream mediators of TGF-β signaling also display symptoms overlapping with TGFBR mutation syndromes. A mutation in Smad3 was recently linked to a heritable syndrome of vascular aneurysms, arterial tortuosity (twisted, corkscrew like arteries), skeletal/craniofacial abnormalities, and osteoarthritis that is referred to as Aneurysm-Osteoarthritis syndrome (AOS) [71]. Similarly, arterial tortuosity

syndrome (ATS), characterized by tortuosity of medium to large vessels and aneurysms, has associated with loss-of-function mutations in SLC2A10 (Solute Carrier Family 2, Facilitated Glucose Transporter Member 10) [72, 73]. Increased TGF-β signaling is also believed to be associated with this syndrome, as ATS vascular smooth muscle cells exhibit decreased production of decorin, a large extracellular leucine rich proteoglycan that is known to bind and sequester TGF-β in the ECM [73]. The decorin promoter contains a glucose response element which is less active with fewer functional glucose transporters, creating a decorin deficiency that results in increased TGF-β abundance and signaling [73]. While these two syndromes further implicate TGF-β signaling through similar connective tissue and cardiovascular phenotypes, syndromes such as Shprintzen-Goldberg syndrome and hereditary hemorrhagic telangiectasia illustrate the wide variation in connective tissue and cardiovascular involvement possible with mutations in TGFBRs.

8.3.2 Shprintzen-Goldberg Syndrome (SGS)

In 1982, Shprintzen and Goldberg first described their eponymous heritable connective tissue syndrome in two patients [74]. Shprintzen-Goldberg syndrome is characterized by anomalies of the head/face, skeleton, brain, and cardiovascular system [75]. Shprintzen-Goldberg syndrome has since been recognized as part of a group of phenotypically overlapping syndromes associated with TGFBR mutations (LDS and FTAAD) affecting connective tissues and the cardiovascular system [76]. However, SGS has been linked to mutations in TGFBRI and II, as well as fibrillin-1 (OMIM #182212). Thus, unlike LDS and MFS, it is not yet known whether the connective tissue and cardiovascular manifestations of SGS are due to a defect in a TGFBR (causing dysregulated TGF-β signaling that results in tissue defects) or a connective tissue component like fibrillin-1 (a structural defect causing dysregulated TGF-β signaling). Independent of the initiating event, the defect lies somewhere in the TGF-β pathway creating a heterogeneous range of symptoms, making a definite genotype-phenotype correlation difficult. Consequently, the clinical presentation of SGS is not well defined and still developing. Intellectual impairment may be the only regularly occurring symptom, with all documented patients presenting with a range from moderate retardation to learning disabilities [77]. These impairments are known to occur simultaneously with brain abnormalities: communicating hydrocephalus, dilated lateral ventricles, and Arnold-Chiari formation type-I [78]. Ocular defects may also be present in SGS patients. Lens dislocation, while seen in MFS, does not appear to be a typical feature of SGS [8]. Hypertelorism (seen in LDS), myopia and exophthalmos, however, are characteristic of SGS [75]. Several skeletal anomalies are identified in early childhood [79]. The major characteristic skeletal finding is scaphocephaly (boat shaped skull) with craniosynostosis (premature fusion of skull) [79]. In fact, SGS has been referred to as marfanoid habitus with craniosynostosis [80].

Many of the skeletal findings associated with MFS and LDS are observed in SGS: dolichostenomelia (long limbs), arachnodactyly (long fingers), scoliosis, pectus excavatum or carinatum (hollowed or pigeon chest), joint hypermobility, and contracture of the proximal joints of the hand [59]. Regarding facial dysmorphic features, SGS may produce micrognathia, midface hypoplasia, low-set ears, and palatal soft tissue hyperplasia (pseudocleft palate) that may be noted as early as the first year of life and become more pronounced with time [77]. Additional characteristic findings include: minimal subcutaneous fat, hypotonia, obstructive apnea, defects in the abdominal wall musculature with hernias, hyperelastic skin, and cryptorchidism [77].

Cardiovascular defects are mostly limited to the heart valves. Mitral and/or aortic valve regurgitation is commonly observed [75]. Mitral valve prolapse, often seen in MFS, occurs commonly as well [77]. Mitral valve prolapse (MVP) and regurgitation are commonly found in many but not all patients with syndromes related to TGFBR mutations. Given that FBN1 mutations have been

associated with an increase in TGF-β release and signaling, Ng et al. examined the association of TGF-β pathway signaling with the pathogenesis of MVP using a mouse model of MFS. Changes in mitral architecture were observed to be temporally and spatially linked with increases in TGF-β activation, signaling and cell processes within the mitral valve (increased proliferation/growth and decreased apoptosis) [81]. Furthermore, normal valve phenotype was restored with the administration of a TGF-β neutralizing antibody. This study provided a potential pathogenic mechanism for MVP in MFS/LDS and perhaps SGS, as mutations in TGFBRs have been linked to increased markers of TGF-β signaling.

Aortic root dilatation and aneurysm has been previously described in SGS, but is not present in most affected individuals, though it is common in MFS, LDS, and FTAAD [8]. The presence of aortic dilatation may suggest overlap with one of these phenotypically similar syndromes. Aortic valve pathology has also been linked to TGFBRII mutations. An SGS patient with a BAV and an ascending aortic aneurysm that later dissected was found to have a mutation in TGFBRII [82]. Thus, both the mitral and aortic valvular manifestations of SGS may be due to mutations in TGFBRs that result in increased TGF-β pathway signaling. While SGS displays a high ratio of connective tissue to cardiovascular symptoms, hereditary hemorrhagic telangiectasia (HHT), another hereditary disorder associated with TGFBR mutations, displays primarily diffuse vascular symptoms.

8.3.3 Hereditary Hemorrhagic Telangiectasia (HHT)

Originally described in the nineteenth century by Osler, Weber, Rendu and Hanes, HHT is an autosomal dominant disorder characterized by vascular malformations and dilated small blood vessels which are fragile due to thin supporting connective tissue [83–85]. HHT is most commonly caused by mutations within TGFBRs that disrupt normal TGF-β signaling, which induces the characteristic vascular and connective tissue defects. Epidemiologic reports estimate the prevalence of HHT between 1 in 5,000 and 1 in 8,000, though some reports believe HHT may be underreported due to many patients being unaware of their diagnosis [86–88]. The diagnosis is often difficult due to its variable penetrance and severity, as well as its relatively slow progression. Manifestations of HHT typically are not present at birth and develop with time. Clinical signs and symptoms may be present in childhood though generally are noted after puberty with an estimated 7 in 10 HHT patients developing at least one clinical symptom or sign by age 16 and almost 100 % by 40 years of age [89–91].

Initially, HHT patients will develop telangiectasias, small blood vessels that dilate near the surface of the skin, mucous membranes and gastrointestinal tract. These telangiectasias increase in number and size with age [92, 93]. Nosebleeds (also known as epistaxis), the most common clinical manifestation of HHT, result from ruptured telangiectasias of the nasal mucosa. Epistaxis and telangiectasias within the gastrointestinal tract, commonly in the duodenum, are the two major mechanisms of iron deficiency anemia secondary to hemorrhage in this population. Most HHT patients experience only these three symptoms: nosebleeds, mucocutaneous telangiectasias, and iron deficiency anemia. These symptoms are relatively minor, in terms of their contribution to the morbidity and mortality associated with HHT, while the primary concern results from vascular abnormalities resulting from malformed connections between arteries and veins in the visceral organs [86].

While arteriovenous malformations (AVMs) may occur sporadically in the general population, AVMs occur in high numbers in multiple organs in HHT patients. The most clinically relevant locations are distributed among the lungs (50 %), the liver (30 %) and the brain (10 %) [94–96]. A further pulmonary manifestation of HHT is severe pulmonary artery hypertension (PAH) arising mainly from two sources in HHT: (1) high output heart failure secondary to hepatic AVM shunting and (2) primary PAH without signs of heart failure [97].

Additionally, HHT patients may also exhibit pathologic defects within specialized connective tissues such as the blood and the immune system/lymphoid tissue. Elevated clotting factor VIII and von Willebrand factor were measured in the blood of HHT patients versus normal controls and associated with venous thromboembolism [98]. Reports of defects in adaptive immunity and a mononuclear cell infiltrate around telangiectases spawned a suspicion of immune system involvement in HHT. These reports were further supported by an analysis of the oxidative burst activity of HHT monocytes and polymorphonuclear cells, which found single or multiple deficits in both cell groups in 20 of 22 HHT patients [99]. Thus, the connective tissue component of HHT has only recently been demonstrated.

The Curacao diagnostic criteria are based on international consensus and used to diagnose HHT with a score that gauges the likelihood of its presence [100, 101]. The criteria include a first degree relative with HHT, the presence of several telangiectasias on the skin and mucous membranes, recurrent and spontaneous epistaxis, and visceral AVMs. One point is scored for each of the criteria present. If only 1 of the criteria is noted, HHT is "unlikely." Two criteria indicate "suspected" HHT. More than two criteria present is evidence of "definite" HHT disease. The diagnosis of HHT is made clinically, without requiring genetic testing to identify a potentially causative mutation. If desired, genetic testing may be employed to confirm the diagnosis.

At least five genes have been identified in which mutations will cause HHT. These are subdivided based on the gene loci involved [97]. HHT1 and 2 are the major subtypes linked to mutations within endoglin (a type-III TGFBR, OMIM #187300) [102] and activin receptor-like kinase 1/ALK-1 (a type-I TGFBR, OMIM #600367) [103, 104]. Additionally, mutations in Smad4 produce HHT with juvenile polyposis. Interestingly, juvenile polyposis in the general population results from mutation in activin receptor-like kinase 3/ALK-3 (also known as BMP Receptor 1A), which signals through Smad4 [97]. HHT producing mutations within downstream proteins involved in TGF- β signaling further confirms the role of TGF-β dysregulation in the pathogenesis of HHT. Of note, a family with juvenile polyposis, aortopathy and mitral valve dysfunction co-segregating with a Smad4 mutation has recently been described [105]. Aortopathy and mitral valve defects are typical features of MFS and LDS, not HHT (though case reports of large vessel aneurysms in HHT exist) [105]. The presence of these features associated with a Smad4 mutation supports the role of TGF-β signaling in the common pathogenesis of all of these features, particularly noncanonical signaling given the role of Smad4 in the canonical pathway. Furthermore, it provides a spectral link between the vascular features (aortopathy, aneurysm, and mitral valve defects) of MFS and LDS and those seen in HHT (AVMs, small vessel dilatation, and juvenile polyposis).

Activin receptor like kinase 1 and endoglin are expressed on the surface of vascular endothelial cells, suggesting that dysregulated TGF-β signaling in endothelial cell plays a major role in inducing telangiectasia/dilatation and AVM formation [106]. Interestingly, homozygous ALK-1 mutations in zebrafish and mice produce embryonic lethality and exhibit severely dilated vessels (including the aorta) and abnormal vessel fusion [107, 108]. These vascular defects were associated with increased endothelial cell number, enhanced expression of angiogenic factors and proteases, and deficient differentiation and recruitment of smooth muscle cells. Thus, the small vessel dilatation in HHT represents a phenotypic microcosm of the aortic and extra-aortic dilatation seen in MFS and LDS. Furthermore, Seki et al. demonstrated in mice, that ALK-1 is predominantly expressed in the developing endothelium of arteries [109]. Taken together, these observations support the role of TGF-β signaling in early vascular development and dilatation.

Transforming growth factor-β can signal through two distinct type-I receptors (ALK-1 and TGFBRI) in endothelial cells [43]. Signaling through TGFBRI activates Smads2/3, while ALK-1 is unique among type-I receptors in that it activates Smads 1, 5 or 8 (Fig. 8.1). Endoglin interacts with ALK-1 and is required for TGF-β-dependent ALK-1 signaling by facilitating the

binding of TGF-β to the ALK-1 receptor [43]. TGF-β stimulation of the endoglin/ALK-1 pathway via Smads 1, 5, or 8 is associated with endothelial proliferation and migration-essential to angiogenesis, while signaling through the ALK-5 pathway via Smad2/3 produces opposite results [110]. This would seem to suggest that mutated ALK-1 or endoglin would result in a quiescent endothelium, opposite to that seen in HHT. However, the ALK-1 pathway regulates the expression of ALK-5 such that decreases in ALK-1 signaling produce a reduction in ALK-5 signaling as evidenced by an 80 % decrease in ALK-5 RNA transcripts in HHT 1 and 2 endothelial cells [111]. This adaptive compensation may produce an imbalance favoring dysregulated angiogenesis and the formation of AVMs. Evidence suggests that the ratio of ALK-1 to ALK-5 determines whether or not endothelial cells will become quiescent or actively proliferate and migrate [112]. Alternatively, similar to LDS in which increased TGF-β signaling was described despite TGFBRI and II mutations, endoglin and ALK-1 mutations may paradoxically increase signaling through angiogenic pathways thus resulting in AVMs.

In comparison to what is known about the mechanisms of AVMs and dilatation, little was known about the mechanism of primary PAH in HHT until recently. The primary PAH phenotype (without heart failure) observed in <2 % of HHT patients is identical to inherited primary PAH due to a loss of function mutation in Bone Morphogenetic Protein receptor 2 (BMPR2), another receptor of the TGF-β superfamily [97]. The mechanism is thought to involve a loss of pulmonary artery endothelial and smooth muscle cell apoptosis mediated by BMPR2 that results in abnormally elevated growth and proliferation [113–115]. Accordingly, the linkage between BMPR2 and ALK-1 was studied in HHT families with PAH and suggestive linkage was found [116]. However, the HHT patients only exhibited mutations in ALK-1, not BMPR2. This suggests a common signaling pathway downstream of BMPR2 and ALK-1 is involved in the pathogenesis of primary PAH. Endoglin, as a type-III TGFBR, facilitates signaling through ALK-1, and endoglin mutations could also result in PAH. Transforming growth factor-β receptor III, also known as betaglycan, has been implicated in the regulation of muscle cell proliferation and was demonstrated to inhibit myoblast proliferation through increasing Smad3 and p38 MAPK signals [107, 117]. Thus, interrupted TGF-β signaling via a mutated ALK-1 or endoglin gene may remove a TGF-β mediated growth inhibitory effect on vascular endothelial or smooth muscle cells and contribute to the development of PAH and other cardinal manifestations of HHT-telangiectasias and AVMs.

8.3.4 Other Connective Tissue Disorders with TGF-β Involvement

TGF-β is also implicated in several other connective tissue disorders which are not commonly defined by gene abnormalities. Most include a hyperactive immune system as a component and are referred to as autoimmune connective tissue diseases. These diseases include systemic sclerosis/scleroderma, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE). Scleroderma (a disease of excessive fibrosis of vessels, organs and particularly the skin) severity has been correlated with levels of TGF-β, a potent regulator of ECM deposition [118]. Rheumatoid arthritis patients have elevated plasma thrombospondin-1 and TGF-β; the former activates TGF-β and the latter induces the expression of connective tissue growth factor (CTGF), which has been associated with atherosclerosis, a process occurring prematurely in RA [119, 120]. Recent evidence has identified a role for TGF-β in suppressing immune function directly and stimulating T cell conversion to a suppression phenotype [121]. Interestingly, TGF-β production is decreased in SLE [122]. Thus, a lack of TGF-β may contribute to SLE through diminished ability to suppress the immune system [123]. Due to its relationship with fibrosis and immune modulation, TGF-β may plausibly be involved in all autoimmune connective tissue disorders though its exact roles remain to be clarified.

8.4 Current Standard of Care for TAAs and Genetic Testing

8.4.1 Current Standard of Care for TAAs

The prognosis of aortic aneurysms has improved substantially with evolving surveillance, medical and invasive management. Consensus guidelines developed by several medical profession organizations for aortic disease with specific syndrome recommendations were published in 2010 [124]. Routine vascular imaging is the mainstay of prognosis (predicting rupture) with all aortic aneurysms. Patients should undergo aortic diameter screening immediately upon diagnosis of a TAA or gene mutation associated with TAA (TGFBRI or II, FBN1, Ehlers-Danlos and FTAAD genes) [124]. Follow-up imaging should be done 6 months later to determine the rate of progression of the aneurysm, with increasing diameter associated with increasing rupture risk [124]. The aortic diameter is followed with at least annual imaging until the rupture risk outweighs the risks of aortic replacement surgery, which is the definitive treatment for TAA. Special consideration is also given if valve or cardiac function is impaired. Rapid progression (defined as > or = 1 cm/year) or a diameter between 5 and 6 cm or 6 and 7 cm in the ascending and descending aorta, respectively, indicates the need for surgery in normal patients [125–128]. For patients with aortic aneurysm and a mutation in TGFBRI or II, guidelines recommend surgery at an external diameter of the ascending aorta >4.4 cm given that rupture and/or dissection is known to occur at smaller diameters in this population [59, 124]. Due to the tendency of patients with these mutations to develop cerebral and abdominal aneurysms, annual MRIs from the head to pelvis are also recommended.

The primary surgical treatment of those with TAA and aortic aneurysms/dissections in general is aortic replacement with artificial grafts. The approach, techniques, and risks involved depend greatly on the location of the aneurysm along the aorta. The major risks involved with an open surgical procedure include death, stroke, paresis/paralysis and renal failure [129, 130]. Endovascular aortic repair (EVAR) is a recent advancement employing stent-grafts to isolate aneurysms from blood flow and pressure/tension that has reduced early post-operative morbidity and mortality compared to open surgery. Long-term observation has revealed late complications related to the EVAR procedure and stent-grafts, provoking doubts about the durability of EVAR [131, 132]. Additionally, this new technique is limited to only 20 % of TAA patients due to its applicability only at certain anatomical sites [133, 134]. Thus, replacement surgery remains the gold standard of care [135, 136].

To delay TAA progression, medical treatment focuses on reducing blood pressure and therefore aortic wall tension, with beta blocker therapy as the current standard of care. However, a clinical trial has suggested that angiotensin converting enzyme (ACE) inhibitors are superior to beta blockers, demonstrating reduced aortic stiffness, improved distensibility, and reduced increase in aortic root diameter [137]. Angiotensin II, a circulating protein that regulates vascular smooth muscle tone, is synthesized by ACE and exerts its effects via two receptor types, ATI and II. Interestingly, ATI stimulation has been linked to enhanced expression of TGF-β and its receptors as well as the promotion of vascular fibrosis [138]. In mice, treatment with losartan, an ATI-specific inhibitor, prevented aneurysm development [139]. Additionally, Habashi et al. noted that mice heterozygous for FBN1 mutation and ATII deficient displayed worse aortic disease and decreased survival compared to mice with only FBN1 mutation, suggesting stimulation through ATI is deleterious and stimulation through ATII is beneficial [140]. When the ATII deficient/heterozygous FBN1 mice were compared to mice with only FBN1 mutation, enhanced phosphorylation of extracellular-signal regulated kinase (ERK) 1 and 2 was noted in the ATII receptor deficient/heterozygous FBN1 mice [140]. After noting increased TGF-β and increased pERK1/2 in FBN1 mutation only mice, this group demonstrated that aortic disease was improved by

stimulation of ATII receptors to decrease ERK1/2 phosphorylation, partially explaining the mechanism of benefit of losartan treatment in early clinical trials [140]. Furthermore, losartan blockade was also shown to rescue the MFS-dependent skeletal muscle defects (impaired regeneration phenotype) and improve alveolar septation defects in murine models [139, 141]. Besides ERK1/2, other noncanonical pathways have also been implicated in MFS mouse models of aneurysm. In Smad4 deficient MFS mice, like the ATII deficient MFS mice, aortic disease was exacerbated and accelerated death associated with elevated c-Jun N-terminal Kinase-1 (JNK1) activation was observed [142]. Interestingly, antagonism of JNK1 attenuated aortic growth in MFS mice with and without Smad4 [142]. As enhanced noncanonical TGF-β signaling is increasingly associated with MFS aortic pathology, ATI, ERK1/2 and JNK1 blockade represent potentially new standards of care for MFS and possibly other TGFBR mutation syndromes. As new pathway-specific treatment modalities are developed, identification of the causative mutation by genetic testing will be increasingly important in cases of inherited aortopathies.

8.4.2 Genetic Testing

8.4.2.1 Genetic Testing for FTAAD

Due to its phenotypic overlap with other inherited aortopathies, FTAAD should be confirmed with genetic testing. Genetic testing in cases of inherited aortopathy can be beneficial in several ways. Identification of an associated mutation can change follow up and medical management of the affected patient. Furthermore, identification of the mutation present in the proband will narrow and facilitate testing in potentially affected relatives as well as with prenatal testing. Beginning with identification of the first family member (proband) with an FTAAD mutation, guidelines recommend all first degree relatives be genetically counseled and screened [143]. Those relatives found to have the genetic mutation should obtain baseline aortic imaging immediately, and second degree relatives could reasonably be notified. If aortic disease is found in any first degree relatives, imaging of second degree relatives would be warranted [143]. If a patient with aneurysm/dissection does not have any of the major gene mutations associated with heritable aortic disease, first degree family members are recommended to seek aortic imaging rather than genetic testing [143]. This recommendation is particularly relevant because only ~25 % percent of FTAAD patients will have one of the seven known associated mutations [54].

Prenatal testing is possible, but it cannot be used to determine the severity of disease in a child inheriting any of the mutations described in this chapter. In addition, limited outcome data on the benefits of genetic testing in patients with heritable aortopathies is available. Given the good longevity of patients with heritable aortic disease with medical treatment and surgery, prenatal testing could reasonably be delayed until childhood, though this issue ideally should be discussed by the parents prior to conception.

8.4.2.2 Genetic Testing for SGS

SGS is clinically suspected when an individual presents with a combination of the major characteristics: marfanoid skeletal features, craniosynostosis, craniofacial dysmorphism, left sided heart valve prolapse or regurgitation, intellectual disability with delayed milestones, and brain abnormalities [75]. No specific diagnostic criteria or scoring rubric exists for SGS as for MFS with the Ghent criteria and HHT with the Curacao criteria. Genetic diagnosis of SGS is difficult due to the limited number of SGS patients, the range of mutations associated, and phenotypic overlap of related syndromes –MFS and LDS- known for their variability in presentation. Fibrillin-1 mutations were initially reported in three clinically diagnosed SGS patients, two of whom had a mutation atypical of MFS and exhibited an overlapping phenotype between SGS and MFS [144, 145]. A later genetic study of multiple SGS patients found no FBN1 mutations [80]. These observations suggest that a similar signaling pathway is involved in both SGS and MFS even though more than one gene may be affected. A patient described by van Steensel et al. with a

TGFBR2 mutation displayed a phenotype overlap between SGS and LDS [146]. In a study describing TGFBR mutation phenotypes, a TGFBR1 mutation was identified in a patient with clinically diagnosed SGS [147]. Currently, no specific mutations have to be identified to diagnose SGS, though identification of an FBN1 or TGFBR mutation may suggest it.

8.4.2.3 Treatment and Genetic Testing for HHT

In those cases where HHT is symptomatic, the management is the typical treatment of each of the individual conditions as though they occurred in a patient without HHT. AVMs are treated based on their location by clinicians with expertise pertaining to the organ involved, HHT or both. Embolotherapy is the preferred and hopefully definitive therapy for AVMs [88], though surgical resection or arterial ligation are further options [101]. If treatment of hepatic AVMs is not successful, the only definitive therapy remaining that will prevent the dire complications of hepatic AVMs (e.g., heart failure, pseudocirrhosis, and portal hypertension) is hepatic transplantation [148, 149].

HHT presents typically in childhood with a progression of subtle clinical signs increasing with age. Therefore, whether a newborn has inherited HHT cannot possibly be determined clinically, and as result genetic testing becomes essential for this purpose [150]. Today, genetic tests examine endoglin, ALK-1 and Smad4 whose mutations are responsible for more than 80 % of HHT cases [97]. However, this means the diagnosis for a substantial portion (~20 %) of individuals with HHT symptoms cannot be confirmed or excluded by molecular genetic testing. With regard to prenatal genetic testing for HHT, little impetus exists for it because of HHT's longevity and relatively asymptomatic course of most patients. In most instances, a positive genetic test does not alter the recommended treatment and screening course of HHT. However, if a Smad4 mutation (associated with juvenile polyposis) is detected in a HHT patient with a family history of gastrointestinal (GI) polyps and/or malignancy, more rigorous GI screening is recommended to reduce the risk of GI cancers [151]. As noted previously, few people diagnosed with HHT know they have it and genetic counseling offers the opportunity to address this communication deficiency directly with patients. For these reasons, genetic testing and counseling are of great potential benefit to HHT suspected and affected families.

8.5 Summary

We have reviewed several heritable connective tissue syndromes associated with mutations in TGF-β receptors I and II as well as accessory receptors and related proteins. Significant progress has been made in understanding their underlying mechanisms, and with their refinement, the probability for insights yielding treatment strategies increases. In particular, the study of TGFBR mutation syndromes holds great promise in this regard for the treatment of both connective tissue and cardiovascular disorders. Mutations in FBN1 or TGFBRs appear to result in a number of phenotypically overlapping connective tissue/cardiovascular syndromes involving dysregulation of the TGF-β signaling pathway. These TGF-β dysregulation syndromes (MFS, LDS, FTAAD, HHT, SGS, AOS and ATS) exhibit a spectrum of cardiovascular defects including arterial aneurysm/dilatation, dissection, mitral valve disease, arterial tortuosity, and primary PAH (Fig. 8.2). Their pathogeneses emphasize a common theme, that normal TGF-β family signaling is integral to the normal development and homeostasis of connective tissues and the cardiovascular system. This family contains potent regulators of many cell types within mesoderm-derived tissues. Thus, perturbations within their signaling pathways are uniquely situated to produce defects in these tissue types.

Clearly, normal TGF-β signaling is essential in angiogenesis. This is certainly evident in hereditary hemorrhagic telangiectasia (HHT), a small vessel phenocopy of the dilatation seen in large vessels of MFS, LDS, FTAAD, ATS and AOS. Angiogenesis, though, not only involves the proliferation and migration of endothelial cells to form new vessels but also smooth muscle cells

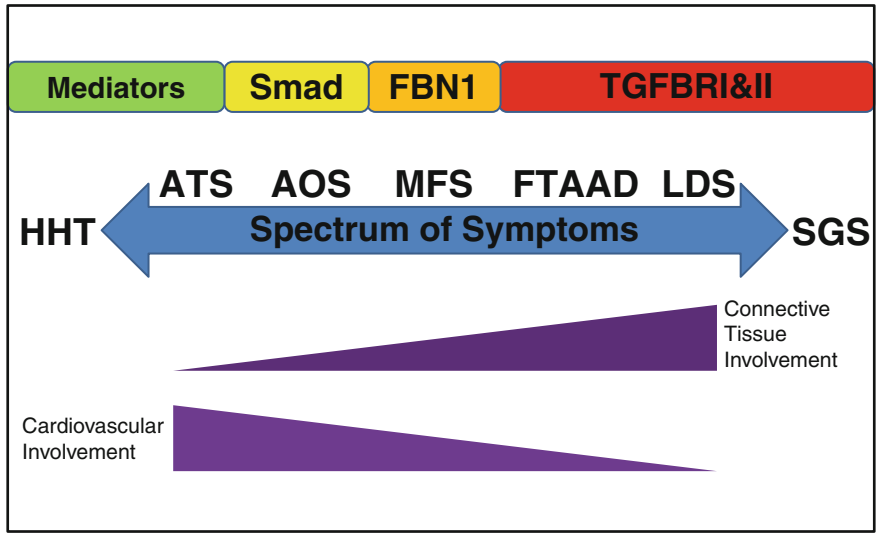

Fig. 8.2 Known heritable connective tissue disorders with cardiovascular involvement that associate with gene mutations related to TGF-Beta signaling. These disorders are arranged based on their increasing level of either connective tissue or cardiovascular involvement and notably share a spectrum of common symptoms, which supports their related pathophysiology

and fibroblasts to strengthen and support these new vessels. Fibroblasts in particular are major cellular regulators of connective tissue homeostasis. Angiogenesis is certainly a process of coordinating endothelial cell behavior in concert with the behavior of fibroblasts and smooth muscle cells. Although many of the mechanisms by which this coordination occurs have yet to be delineated, the TGF-β superfamily is highly implicated in regulating the normal behavior of all of the cells involved.

Many major connective tissue syndromes including Marfan syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos syndrome exhibit concomitant cardiovascular manifestations. In Marfan syndrome and its related disorders, characteristic abnormalities of these syndromes that were once thought to result from purely structural deficiencies (e.g. FBN1) are now attributed to disruptions of normal TGF-β signaling. The undeniable overlap in connective tissue and cardiovascular phenotypes of fibrillinopathies and mutated TGF-β receptor syndromes supports this notion of a common signaling pathway [8]. Indeed, these mutated receptor phenotypes are even recapitulated by mutations of downstream TGF-β pathway components (e.g. Smad4 in HHT and Smad3 in LDS and FTAAD) [71, 151].

Our new understanding of causal signaling disturbances in these disorders significantly improves the treatment prospects for highly morbid cardiovascular and debilitating connective tissue defects beyond the difficult prospect of restoring structural integrity to weakened tissues. Recently, additional heritable connective tissue disorders-cutis laxa and congenital contractural arachnodactyly-have been linked to dysregulated TGF-β and BMP signaling.[152, 153] Perhaps, those heritable connective tissue syndromes currently attributed to structural ECM weakness (e.g., osteogenesis imperfecta) will also be revealed to involve disturbances in signaling pathways, boding well for the development of future medical treatments.

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The Ehlers-Danlos Syndrome

9

Fransiska Malfait and Anne De Paepe

Abstract

The Ehlers-Danlos Syndromes comprise a heterogeneous group of diseases, which are characterized by fragility of the soft connective tissues and widespread manifestations in skin, ligaments and joints, blood vessels and internal organs. The clinical spectrum varies from mild skin and joint hyperlaxity to severe physical disability and life-threatening vascular complications. The current Villefranche classification recognizes six subtypes, most of which are linked to mutations in one of the genes encoding fibrillar collagen proteins or enzymes involved in post-translational modification of these proteins. Establishing the correct EDS subtype has important implications for genetic counselling and management and is supported by specific biochemical and molecular investigations. Over the last years, the characterisation of several new EDS variants has broadened insights into the molecular pathogenesis of EDS by implicating genetic defects in the biosynthesis of other extracellular matrix molecules, such as proteoglycans and tenascin-X, or genetic defects in molecules involved in intracellular trafficking, secretion and assembly of extracellular matrix proteins.

Keywords

Ehlers-Danlos syndrome (EDS) • Villefranche classification • Six subtypes • Non-functional *COL5A1* allele • *COL3A1* gene • Beighton hypermobility score • *COL1A1* and *COL1A2* mutations

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Abbreviations

ATCS	Adducted Thumb Clubfoot Syndrome
ECM	Extracellular Matrix
EDS	Ehlers-Danlos Syndrome
ER	Endoplasmic Reticulum
HP	Hydroxylysylpyridinoline
JHS	Joint Hypermobility Syndrome
LP	Lysylpyridinoline

9.1 Classification

The Ehlers-Danlos syndrome (EDS) comprises a spectrum of monogenic conditions with multisystemic and variable clinical manifestations affecting primarily the skin, ligaments and joints, blood vessels and internal organs. Like Osteogenesis Imperfecta, EDS represents a paradigm collagen disorder among the larger group of heritable connective tissue diseases. Genetic defects affecting the biosynthesis or structure of collagen type I, III and V have currently been implicated in EDS and form the basis of the 1997 Villefranche classification of EDS, which recognizes six subtypes, based on clinical phenotype, inheritance pattern and underlying biochemical and molecular defect(s) [1] (Table 9.1). The classic, hypermobility and vascular subtype of EDS are the most common, whereas the kyphoscoliosis, arthrochalasis and dermatosparaxis types constitute very rare conditions. For each of these subtypes a set of major and minor diagnostic criteria has been defined. Over the last years, the clinical and molecular delineation of several new EDS variants has called for an expansion of the current classification and also demonstrated that, besides the collagens, genetic defects affecting either the biosynthesis of other extracellular matrix (ECM) components or processes as diverse as signalling pathways or intra-cellular trafficking can contribute to EDS pathogenesis.

Table 9.1 Updated EDS-classification

EDS-subtype	Inheritance pattern	Protein	Gene
Classic	AD	Procollagen type V	*COL5A1/COL5A2*
		Procollagen type I	*COL1A1*
	AR	Tenascin-X	*TNX-B*
Cardiac-valvular	AR	Deficiency of α2(I) collagen chain	*COL1A2*
Hypermobility	AD	?	*?*
		Tenascin-X	*TNX-B*
Vascular	AD	Procollagen type III	*COL3A1*
Vascular-like	AD	Procollagen type I (R-to-C)	*COL1A1*
Kyphoscoliotic	AR	Lysyl hydroxylase-1	*PLOD1*
Musculocontractural	AR	Dermatan-4-sulfotransferase-1	*CHST14*
Progeroid	AR	Galactosyltransferase 1	*B4GALT7*
Spondylocheirodysplastic	AR	ZIP13	*SLC39A13*
FKBP14	AR	FKBP14	*FKBP14*
Brittle cornea syndrome	AR	ZNF469	*ZNF469*
		PRDM5	*PRDM5*
Arthrochalasis	AD	Procollagen type I (deletion of N-propeptide cleavage site)	*COL1A1/COL1A2*
EDS/OI overlap	AD	Procollagen type I (delay in N-propeptide cleavage)	*COL1A1/COL1A2*
Dermatosparaxis	AR	Procollagen-I-N-proteinase	*ADAMTS2*

AD autosomal dominant, *AR* autosomal recessive

9.2 General Clinical Manifestations of EDS

The main clinical characteristics listed below are present in varying degrees in each subtype of EDS. One of the most typical features is the ***skin hyperextensibility,*** which means that the skin stretches easily but snaps back after release (unlike lax skin in "cutis laxa" syndromes). The skin is often smooth and velvety to the touch. In the vascular subtype, the skin is not hyperextensible but thin and transparent, showing a prominent venous pattern. The skin is ***fragile*** and splits easily after minor trauma especially over pressure points and exposed areas, which typically show widened and thin ***atrophic scars***, referred to as 'cigarette paper scars'. ***Joint hypermobility*** is usually generalised and variable in severity and with age. It is assessed using the Beighton scale (Table 9.2). While often an innocent 'asset' in childhood and adolescence, it can become a serious burden over time, often complicated by repetitive (sub)luxations, sprains and chronic, debilitating joint pain that is difficult to treat and may lead to devastating physical, social and emotional disability. Muscle hypotonia may cause delay in motor development, problems with ambulation and mild motor disturbance. ***Easy bruising*** is common, manifesting as spontaneous ecchymoses and hematomas that often recur and may cause unaesthetic discoloration of the skin due to hemosiderin deposits in exposed areas such as shins and knees. There is a tendency towards pronounced ***bleeding*** (e.g. following brushing of teeth) despite a normal coagulation status. A range of clinical manifestations that result from ***generalized weakness and fragility of the soft connective tissues*** are observed in patients with EDS including obstetrical and gynaecological complications such as cervical insufficiency, premature rupture of membranes, vaginal tears and lacerations, surgical complications such as wound dehiscence and incisional hernia, tissue prolapses, umbilical or hiatal hernia.

9.3 EDS-Subtypes

9.3.1 Ehlers-Danlos Syndrome, Classic Type

The diagnosis of the classic type of EDS requires the presence of skin hyperextensibility, widened atrophic scars and joint hypermobility, which constitute the three major diagnostic criteria, in association with a varying set of 'minor' manifestations such as smooth, velvety skin, molluscoid pseudotumours (fleshy lesions over pressure points), subcutaneous spheroids (small, hard cyst-like nodules), easy bruising and bleeding, muscle hypotonia, delayed gross motor development and inguinal and/or umbilical hernia. Characteristic facial features include epicanthic folds, excess skin over the eyelids, presence of one or more dilated scars on the forehead and a pale, somewhat prematurely aged appearance of the face.

Ultrastructural examination of the skin in classic EDS shows irregular and loosely packed collagen fibrils and typical "cauliflower" fibrils which represent the histological hallmark of disturbed fibrillogenesis of the heterotypic type I/V collagen fibrils. The molecular basis of classic EDS is a deficiency of ***type V collagen***, a

Table 9.2 The Beighton scale for joint hypermobility. A total score of at least five defines hypermobility

Joint/finding	Negative	Unilateral	Bilateral
Passive dorsiflexion of the 5th finger >90°	0	1	2
Passive flexion of thumbs to the forearm	0	1	2
Hyperextension of the elbows beyond 10°	0	1	2
Hyperextension of the knees beyond 10°	0	1	2
Forward flexion of the trunk with knees fully extended and palms resting on the floor	0	Present = 1	

quantitatively minor fibrillar collagen that is widely distributed in tissues such as skin, bone, tendon, cornea, placenta and foetal membranes. It consists of three different α-chains encoded by the *COL5A1*, *COL5A2* and *COL5A3* gene respectively. The most common isoform in vertebrate tissues is the [α1(V)2α2(V)] heterotrimer. Collagen type V is thought to play a key role in collagen fibrillogenesis via its huge N-propeptide domain that is the only part of the type V collagen molecule that emerges from the surface of the fibrils whereas the entire triple helix domain is buried within the fibril [2].

A causal role for type V collagen in classic EDS first became apparent from studies in transgenic mice which showed that mice with a homozygous deletion of the *col5a2* gene present clinical and ultrastructural features of classic EDS [3]. It was subsequently confirmed by the identification of a (9,X) translocation that disrupted the *COL5A1* gene in a patient presenting with classic EDS and hypomelanosis of Ito [4]. The first mutations reported in classic EDS were respectively an exon skipping mutation [5] and a missense mutation substituting a highly conserved cysteine for a serine in the C-propeptide domain of the α1(V) collagen chain [6]. This cysteine residue is essential for intra-chain disulphide bonding prior to chain assembly and initiation of trimerisation. The mutation prevents incorporation of the mutant collagen chain into the molecule and thus causes a reduction of type V collagen, a mechanism that was subsequently confirmed to be central in the pathogenesis of classic EDS. Since then a growing number of mutations in type V collagen have been identified by different groups, including for the most part heterozygous *COL5A1* nonsense, frameshift or splice site mutations that abolish one *COL5A1* allele through the nonsense-mediated mRNA decay mechanism or impair normal molecular assembly of type V collagen [7–10]. These mutations result in *COL5A1* haploinsufficiency and lead to the production of approximately half the normal amount of type V collagen. A minority of mutations consist of splice site or missense mutations in either *CO L5A1* or *COL5A2* that lead to the production of an abnormal. Polypeptide chain that is incorporated in the molecule and results in the production of structurally abnormal type V collagen molecules. Although to date, approximately 150 different type V collagen mutations have been identified in classic EDS, no particular phenotype-genotype correlations have emerged from these findings, except perhaps for those mutations residing in the highly conserved N-terminal propeptide domain of α1(V) that cause atypical splicing outcome and (have been associated with) a more severe EDS phenotype [11]. Based on the data gathered to date, it is now clear that mutations in type V collagen account for approximately 90 % of classic EDS cases [12].

9.3.2 Ehlers-Danlos Syndrome, Hypermobility Type (EDS-HT)

The exact clinical definition and nosologic delineation of this form of EDS subtype is still a matter of debate and uncertainty, and, since its genetic basis is largely unknown, a precise biomarker or reliable diagnostic test for this EDS subtype is lacking. Moreover, joint hypermobility is a common manifestation in the general population, its phenotypic expression is variable even within families and suitable large families with EDS-HT in which the phenotypic status of all relatives can be unequivocally established on clinical grounds are scarce. Therefore this EDS subtype represents a real diagnostic challenge to the clinician. According to the Villefranche nosology, the major diagnostic criteria are generalized joint hypermobility and typical skin manifestations such as hyperextensibility and smooth, velvety skin, although these are usually much more subtle than in the classic type of EDS. They are nevertheless helpful to differentiate this EDS subtype from the more common '(familial) joint hypermobility syndrome' (JHS). The presence and degree of hypermobility can be scored with the Beighton hypermobility score (Table 9.1) which assesses hypermobility at hands, elbows, knees and spine through different manoeuvres which each are scored with one point to a maximum of nine points [13].

Although often considered in the literature as a 'mild' form of EDS, the hypermobility type of EDS can present with severe and debilitating complications such as recurring dislocations and subluxations and chronic articular pain, which represent a significant burden in daily life of affected individuals and may lead to social isolation, emotional distress and depression [14]. In practice, it is not uncommon to see patients with the EDS-HT diagnosed with fibromyalgia, chronic fatigue syndrome and/or depression.

Over the last years different studies have aimed to document in a more precise way the functional musculoskeletal status and health in patients with EDS-HT. Musculoskeletal symptoms and complaints were shown to be frequently present to a significant degree in EDS-HT patients. Severe joint hypermobility with recurrent joint dislocations and chronic moderate to severe pain were the most severe complaints, but also muscle cramps, tendinitis, headache and fatigue were frequently reported among EDS-HT subjects. Symptoms caused by autonomic dysfunction were reported in more than half of the EDS-HT subjects. These complaints were shown to have a considerable impact on the physical, social and emotional daily life of the EDS-subjects [14]. In a comparative study, physical impairment and impact of joint pain in EDS-HT were shown to be substantially greater in EDS-HT versus other chronic rheumatologic disorders such as rheumatoid arthritis, but rather comparable to the significant disease observed in fibromyalgia [15].

Factors that have been shown to contribute to joint instability include impaired proprioception, postural control and muscular strength. Our studies showed that EDS-HT patients have reduced knee joint proprioception [16], as well as a severely reduced quantitative muscle function and impaired in physical functioning, compared to age and sex-matched controls. EDS-HT patients present lower extremity muscle weakness, which appears not to be caused by reduced muscle mass but rather by intrinsic muscular dysfunction, associated with muscle pain and fatigue [17].

The striking preponderance of affected women versus men in EDS-HT is presently unexplained. Ultrastructural studies have shown that some patients with EDS-HT show collagen fibril abnormalities with cauliflower-like aspect as seen in classic EDS [18]. These findings suggest that somehow, collagen fibrillogenesis is impaired also in this EDS subtype, but so far, besides some anecdotal observations, molecular evidence for this is lacking and the major fibrillar collagens have all been excluded as candidates by linkage studies.

Zweers et al. have demonstrated that a small subset of patients with EDS-HT or JHS shows haploinsufficiency for tenascin-X [19], encoded by the *TNX-B* gene. Tenascin-X is part of a family of ECM proteins with a complex multidomain structure that allows interaction with many other ECM components. It is considered to be a very important player in the organisation of the ECM. An autosomal recessive form of EDS, resembling to but phenotypically distinct from classic EDS [20], had previously been shown to be caused by complete deficiency of **tenascin-X**, caused by truncating mutations or large deletions in both *TNX-B* alleles. Patients with this condition present with joint hypermobility, skin hyperextensibility and easy bruising, but they also suffer from generalized muscle weakness and distal contractures. Atrophic scarring has not been observed. Further discussion of EDS-HT and other less common forms of EDS is also presented in Chap. 10 by Miyake et al.

9.3.3 Ehlers-Danlos Syndrome, Vascular Type

Of all EDS subtypes, the vascular subtype has the worst prognosis because of a propensity to rupture of arteries and hollow organs at young age. Unlike other EDS-types, the skin is not hyperextensible, but rather thin and translucent, showing a visible venous pattern over the chest, abdomen and extremities. Excessive bruising is the most common sign and is often the presenting complaint, especially in children. Other early manifestations include premature rupture of the membranes, congenital clubfoot or congenital hip dislocation, inguinal hernia, recurrent joint dislocation or subluxation and precocious and

severe varicosities. Patients with vascular EDS often display a characteristic facial appearance, with prominent eyes (due to lack of subcutaneous adipose tissue around the eyes), a thin, pinched nose and small lips, hollow cheeks and lobeless ears. Hypermobility is usually limited to the small joints of the hands. Excessive wrinkling and thinness of the skin over hands and feet may produce an old-looking appearance, referred to as "acrogeria". The clinical appearance of patients with vascular EDS may however deviate from the typical picture, and especially the facial and cutaneous features may be very subtle or even absent. In the absence of a positive family history or a major vascular or intestinal complication, early clinical diagnosis can be difficult.

Generalised vascular fragility largely dominates the clinical picture. Apart from excessive bruising and bleeding, it may cause arterial ruptures, potentially resulting in sudden death, usually in the third or the fourth decade of life. Other life-threatening complications include gastrointestinal rupture, rupture of the uterus and internal organ rupture.

A retrospective study, performed on 100 independent, molecularly proven vascular EDS-probands, showed that 7 % of the probands experienced a first major event by the age of 20 years, whereas up to 75 % experienced a first major complication by age 40 years. The vast majority (82 %) of all major complications were arterial. These mostly involved aneurysm, dissection or rupture of medium-sized abdominal vessels (mainly renal, iliac, femoral, mesenteric and hepatic arteries) and/or the abdominal aorta. Other frequent vascular lesions involved carotid, subclavian, ulnar, popliteal and tibial arteries. Coronary rupture, leading to acute myocardial infarction was a rare, but severe complication. Of note, ruptures were not always preceded by detectable aneurysmal dilatation. Presence of a carotid-cavernous fistula was reported in 6 % of the probands. Gastro-intestinal complications accounted for 15 % of the complications, the vast majority of which were spontaneous ruptures of the sigmoid colon, whereas ruptures of the upper gastrointestinal tract were rare. Four probands experienced a spontaneous organ rupture, including spleen or liver. Pneumothorax was a frequent complication. The median age of death was 33 years, and the major cause of death was arterial rupture. Obstetrical history was recorded for 34 pregnancies among which five were complicated by arterial, uterine or splenic rupture. Other pregnancy-or delivery-related complications included severe vaginal lacerations and haemorrhage, and severe rectal tearing. Sixty percent of all probands was referred for molecular analysis only after the occurrence of a major internal complication, such as an arterial or internal organ rupture, whereas the remaining 40 % was referred because of typical clinical manifestations, including excessive bruising, translucent skin, acrogeria and facial appearance, either with or without having a family history of a major event or sudden death. The median age at diagnosis was 29 years, but ranged widely between 4 and 74 years (Malfait and de Paepe, personal observation).

Vascular EDS is caused by heterozygous mutations in the *COL3A1* gene, encoding type III collagen. To date more than 250 *COL3A1* mutations have been identified [21], the majority of which are missense mutations leading to substitutions for glycine in the triple helical region of the collagen molecule. Other mutations include splice site mutations, partial gene deletions, and, rarely, null- mutations resulting in *COL3A1* haplo-insufficiency [22].

Genotype-phenotype correlations have been investigated extensively in vascular EDS. Missense mutations located at the extreme C-terminal end of the molecule usually cause the so-called "acrogeric" form of EDS, associated with severe vascular problems and premature death. This relationship is however not absolute and severe clinical phenotypes have been reported for more N-terminal-located mutations. It was suggested that patients with *COL3A1* null mutations may present a milder phenotype, that is associated with a longer life span, later age of first complication (by 15 years), and risk of complication limited to vascular events [23].

Parental mosaicism for *COL3A1* mutations has been documented in vascular EDS [24–27] and may explain unexpected recurrences in families in which a 'new' dominant mutation has been identified.

9.3.4 Ehlers-Danlos Syndrome, Kyphoscoliotic Type and Related Phenotypes

The ***EDS kyphoscoliotic type or type VIA*** is an autosomal recessive disorder characterized by early onset progressive kyphoscoliosis, severe neonatal muscular hypotonia with delayed gross motor development, generalized joint hyperlaxity, osteopenia, fragile, hyperextensible and bruisable skin, microcornea and in some patients scleral fragility with risk for rupture of the globe, or occurrence of life-threatening rupture of medium-sized arteries. This form of EDS is caused by a deficient activity of the enzyme lysyl hydroxylase 1 (LH-1) [28] and is historically the first EDS subtype for which the molecular defect has been elucidated. LH-1 or PLOD1 hydroxylates lysyl residues in the (Gly-Xaa-Lys) triplets of collagen type I to hydroxylysyl residues, which are involved in the formation of intermolecular cross links (pyridinolines) that provide tensile strength and stability to the collagen fibrils and serve as attachment sites for carbohydrate units which modulate the lateral packing of collagen molecules into fibrils. The diagnosis can be confirmed by demonstrating an increased ratio of lysylpyridinoline (LP) to hydroxylysylpyridinoline (HP) cross-links in the urine, decreased LH-1 activity in cultured skin fibroblasts or a homozygous or compound heterozygous mutation in the *PLOD1* gene. A homozygous multi-exon duplication accounts for ~20 % of mutations reported so far [29], but missense, nonsense and small indel mutations leading to loss-of-function of *PLOD1* have also been identified [30]. Interestingly, in **the *Brittle Cornea Syndrome (BCS)*,** a rare autosomal recessive condition that shows significant phenotypic overlap with EDS type VI A, mutations have been found in *ZNF469,* a gene encoding a Zinc finger protein of unknown function, belonging to the C2H2 Zinc Finger family and expressed in skin, muscle, cornea and sclera [31]. In BCS, thin, brittle cornea and ocular fragility, blue sclera and keratoconus are prominent features but can be associated with skin and joint hypermobility and kyphoscoliosis. Histologic examination of the sclerae shows a significantly decreased corneal thickness. Recently, *PRDM5*, a second gene for BCS was identified [32]. *PRDM5* encodes a widely expressed transcriptional regulator containing 16 C2H2 Zinc Fingers, and has been shown to modulate both canonical and non-canonical Wnt-signaling pathways in early zebrafish development [33]. The phenotypic spectrum of BCS appears to be identical in patients with either *ZNF469* or *PRDM5* mutations, suggesting that the two genes act within the same developmental pathway [32].

A subset of patients who clinically appear to fit within the EDS type VI phenotypic spectrum do present with normal LP/HP ratios and have been referred to as 'EDS type VIB' or 'EDS type VI with normal LH-1 ratios' [34]. We have recently shown that mutations in ***CHST14***, encoding dermatan-4-sulfotransferase 1 (D4ST-1), and previously associated with an autosomal recessive condition called "adducted thumb clubfoot syndrome" (ATCS) (59), underly this EDS type VIB variant [35]. Affected individuals present a range of clinical features that overlap with EDS type VIA, such as the typical skin and joint manifestations, kyphoscoliosis, congenital clubfeet, muscular hypotonia and ocular abnormalities which include microcornea, blue sclera, myopia, retinal detachment and glaucoma. However they also present characteristic clinical manifestations that are distinct from type VIA. These include craniofacial abnormalities, joint contractures, wrinkled palms, tapered fingers and gastro-intestinal and genitourinary manifestations. The craniofacial manifestations and joint contractures are similar to those in ATCS but differ in the severity of the skin manifestations and the more severe kyphoscoliosis and ocular involvement. A Japanese EDS variant reported by Kosho et al. [36] was also shown to be associated with loss-of-function mutations in *CHST14* [37], and falls within this EDSVIB-ATCS spectrum. In view of the prominent muscular hypotonia and typical contractures we have proposed to designate the *CHST14* related EDS variant as the ***EDS, musculocontractural subtype (EDSVIB)***. Morphological and ultrastructural studies in this EDS subtype shows small collagen fibrils with

variable diameter and the presence of flower-like fibrils which are characteristic for EDS. Unlike in EDS type VIA, biochemical collagen studies as well as LP/HP ratios are normal. The molecular spectrum of *CHST14* mutations is varied and comprises homozygous or compound heterozygous missense, nonsense and frameshift mutations, as well as homozygous 20-bp duplication [38].

The identification of mutations in *CHST14* unequivocally links EDS pathogenesis to defects of proteoglycan metabolism. D4ST-1 is a key enzyme in the biosynthesis of dermatan sulfate (DS), where it catalyses 4-O-sulfatation of N-acetyl-galactosamine (GalNAc) residues. It is one of three major sulfotransferases in the DS/CS (chondroitin sulfate) synthesis, which display different substrate specificities. The different epimerisation and sulfatation reactions during DS/CS biosynthesis reflect a tightly controlled system that determines the structural variability and functional interactions of the DS/CS chain. Deficiency of the D4ST-1 enzyme perturbs normal DS/CS balance in DS proteoglycans (DSPG) such as versican and trombomodulin and small leucine-rich proteoglycans, such as decorin and biglycan. This may compromise the functional and structural integrity of these DSPG, which display a widespread tissue distribution and are important in many processes including organization of the ECM, wound repair, anticoagulant processes and cell adhesion. In particular, loss of the normal hybrid DS/CS configuration in decorin may decrease its capacity to regulate the interfibrillar spacing of collagen fibrils and thus lead to disorganisation of the collagen network [37].

Of note, a rare, autosomal recessive condition, **progeroid EDS,** has also been associated with PG biosynthesis. This EDS variant is characterized by progeroid appearance with wrinkled face, curly fine hair and periodontitis in addition to typical features of EDS and caused by homozygous mutations in *B4GALT7,* the gene encoding beta-1, 4 galactosyltransferase or galactosyltransferase I [39]. This enzyme catalyzes the transfer of the first galactose residue on the O-linked xylose in the tetrasacchardie linker region that initiates the biosynthesis of glycosaminoglycans. Very recently, biallelic mutations were identified in *B3GALT6*, which encodes galactosltransferase II, responsible for the transfer of the second galactose residue in this tetrasaccharide linker region, in patients with a pleiotropic EDS-like disorders, presenting also bone fragility, muscle hypotonia, severe kyphoscoliosis and progressive contractures [40, 41] (see also Chap. 10).

These conditions shows phenotypic resemblance with two other recently identified novel autosomal recessive EDS variants. One is the *spondylocheirodysplastic form of EDS***,** which is characterized by hyperextensible, thin skin, easy bruising, hypermobility of the small joints with a tendency to contractures, prominent eyes with bluish sclera, wrinkled palms and atrophy of the thenar muscle and tapering fingers. In addition, patients show moderate short stature and a mild skeletal dysplasia characterized by platyspondyly, osteopenia and widened metaphyses. The total urinary pyridinolines are elevated with a LP/HP ratio of ~1, which is higher than normal values (~ 0,2) but less than in EDSVIA (~6). A homozygous 9 bp deletion in *SLC39A13*, a zinc transporter involved in the intracellular trafficking of Zinc, necessary for normal LH-1 function, has been shown to be causative [42]. The other novel EDS variant is characterized by severe progressive kyphoscoliosis, muscle hypotonia at birth, myopathy, joint hypermobility, hyperelastic skin, sensorineural hearing impairment and normal pyridinoline excretion in the urine. This condition is caused by mutations in *FKBP14*, encoding an endoplasmic reticulum (ER)-resident protein belonging to the family of FK506-binding peptidyl-prolyl cis-trans isomerases. ER-resident FKBPs have been suggested to act as folding catalysts by accelerating cis-trans isomerisation of peptidyl-prolyl bonds and to act occasionally as chaperones. The wide connective tissue involvement in the affected patients is attributed to a disturbance of protein folding in the ER affecting one or more components of the ECM [43].

9.3.5 Ehlers-Danlos Syndrome Subtypes That Result from Aberrant Processing of the Procollagen Type I-N-Propeptide

Defects that interfere with the cleavage of the N-terminal propeptide of type I procollagen result in the *arthrochalasis or dermatosparaxis type of EDS* respectively. The autosomal dominant *arthrochalasis type of EDS* (previously EDS VII A & B) is caused by heterozygous mutations that lead to skipping of exon 6, or part of it, in the mRNA coding for the α1 or α2-chain of type I procollagen. These exon-skipping mutations lead to loss of the N-terminal telopeptide, which links the N- propeptide to the main triple-helical domain and contains the procollagen-I-N-proteinase cleavage site as well as a critical cross-linking lysyl residue. The clinical hallmark of this EDS-variant is congenital bilateral hip dislocation, in addition to severe generalized joint hypermobility with recurrent joint dislocations, variable cutaneous involvement with hyperextensible, bruisable skin, poor wound healing with atrophic scars, muscular hypotonia, kyphoscoliosis and osteopenia. Biochemical confirmation of the diagnosis is based on electrophoretic demonstration of pNα1(I) (EDS VIIA) or pNα2(I) (EDSVIIB) procollagen chains in cultured skin fibroblasts.

Mutations residing within the N-terminal stretch of 85 amino acid (AA) residues in the triple helical domain of type I collagen result in an EDS/OI overlap phenotype characterised by OI-like bone fragility and variable skin- and joint hypermobility, reminiscent of that seen in EDS [44, 45]. This 85 AA region acts as a stabilizing "anchor" for the N-terminal end of the type I collagen triple helix, and defects in this α1(I) N-anchor region were shown to lead to a conformational change in the adjacent N-propeptide cleavage site, resulting in inefficient cleavage of the N-propeptide [46]. So, although the cleavage site itself remains intact, inefficiently cleaved collagen molecules are incorporated in the collagen fibrils, leading to EDS-symptoms by a mechanism similar to EDS type VIIA/B.

Deficient activity of the procollagen-N- proteinase, the enzyme responsible for cleavage of the N-terminal propeptide in type I, II and III collagen and which is encoded by the *ADAMTS2* gene, causes the *dermatosparaxis type of EDS*, an autosomal recessive condition characterized by pronounced skin fragility and a sagging, redundant appearance of the skin. Other distinctive features are delayed closure of the fontanels, characteristic facies with edema of the eyelids and blue sclera, umbilical hernia and short stature. Fragility of internal tissues, with spontaneous bladder and diaphragmatic rupture, has been reported [47]. Whereas most of the initially reported patients showed a very severe phenotype, recognizable from birth, it is now clear that some patients present with a milder condition, which can delay the diagnosis.

As a result of the deficient activity of the procollagen-N-proteinase, uncleaved pN-collagen molecules are incorporated into mature collagen fibrils. This causes pathognomonic abnormalities of the dermal collagen fibril architecture, characterised by fibrils that have lost their normal cross-sectional circular aspect and have a hieroglyphic appearance [48]. Biochemical analysis shows aberrant processing of type I procollagen with characteristic accumulation of type I pN-collagen [49].

9.3.6 Other EDS-Variants Caused by Mutations in the Type I Collagen-Encoding Genes

Over the last years, a number of atypical mutations have been identified in the genes encoding type I collagen (*COL1A1* and *COL1A2*), which have been shown to result in EDS-phenotypes that show overlap with the classic type of EDS. One of those is a rare autosomal recessive form of EDS referred to as the *cardiac-valvular EDS*, caused by total absence of the α2(I) collagen chain which results in the production of $[\alpha1(I)]_3$ homotrimers. This condition presents in childhood with mild skin - and joint hypermobility, mild osteopenia and muscular hypotonia but is complicated in adulthood by the development of severe cardiac valve insufficiency that may need cardiac valve replacement [50, 51].

Another type of type I collagens defects linked to EDS are missense mutations in *COL1A1* that result in the substitution of an arginine (R) residue by a cysteine (C) residue in the Xaa or Yaa position of the triple-helical Gly-Xaa-Yaa repeat. These mutations lead to the production of α1(I)-dimers that are detectable on SDS-PAGE of radiolabelled collagens obtained from cultured skin fibroblasts. One R-to-C substitution in pro 1(I) chain of type I collagen (p. (R312C)) has been identified in a series of patients with classic EDS-like phenotype, showing skin hyperextensibility, easy bruising, atrophic scarring and joint hypermobility, and propensity for arterial rupture at adult age [52, 53]. Two other pro-α1(I) R-to-C substitutions (p.(R574C) and p.(R1093C)) have also been associated with rupture of medium-sized arteries, but affected individuals did not present EDS-like skin features [53]. Furthermore a p.(R1036C) and a p.(R1066C) were reported in families with an EDS/OI overlap phenotype, without signs of vascular fragility [54, 55]. Intriguingly one specific α1(I)_p.(R1014C) substitution was reported in a number of families with autosomal dominant infantile cortical hyperostosis or Caffey disease, a benign and self-limiting disorder of early childhood, characterized by systemic inflammation and subperiosteal new bone formation [56].

9.4 Diagnosis

Comprehensive clinical evaluation is the first and pivotal step in the diagnostic assessment for EDS and may be sufficient to establish a correct clinical diagnosis. For certain EDS-subtypes, ultrastructural, biochemical and molecular analysis are helpful to diagnose the correct EDS subtype.

9.4.1 Ultrastructural Examination of the Skin

Ultrastructural examination of the skin, performed by electron microscopy, usually reveals abnormalities of collagen fibrils. These include irregular, disrupted collagen fibrils ("collagen flowers"), and variability within the diameter of the collagen fibrils. However, these abnormalities are common to several EDS variants and usually not specific enough to discriminate between individual EDS subtypes.

A pathognomonic ultrastructural aspect of the collagen fibril architecture is observed only in the dermatosparaxis subtype of EDS. Collagen fibrils in this EDS subtype lose their normal cross-sectional circular aspect and display an irregular, branched, "hieroglyphic" appearance instead (2).

9.4.2 Skin Biopsy for Fibroblast Culture and Biochemical Analysis

The first step in the laboratory diagnosis of EDS is to establish a fibroblast culture from a skin biopsy. Protein-based analysis of the collagens type I, III and V by means of SDS-polyacrylamide gel electrophoresis allows to detect qualitative or quantitative abnormalities of these collagen proteins.

Biochemical analysis of type III collagen is highly sensitive to confirm a diagnosis of vascular EDS as it identifies structural alterations of the type III collagen proteins in more than 95 % of affected individuals (Malfait et al., in preparation). Of note, *COL3A1* null mutations do not usually lead to a detectable alteration in electrophoretic mobility of type III collagen. Therefore, direct DNA analysis of the *COL3A1* gene is still indicated in clinically suspect cases for vascular EDS, even in the absence of a detectable type III collagen protein defect.

Biochemical analysis is also helpful to confirm the diagnosis of the arthrochalasis, kyphoscoliosis, and dermatosparaxis subtypes of EDS. Rare, unclassified variants of EDS with distinct fibrillar collagen protein abnormalities, such as R-to-C substitutions in the α1(I)-chain of type I collagen, may also be picked up by biochemical collagen analysis. These findings may further guide molecular analysis of the specific collagen gene(s) involved.

In contrast, for the majority of patients with classic EDS, biochemical analysis of type V

collagen is an ineffective method for routine diagnostic evaluation (3).

In the hypermobility type of EDS, as well as in the benign joint hypermobility syndrome (BJHS), biochemical analysis of the fibrillar collagens shows no abnormalities (4).

9.4.3 Urine Analysis

A highly specific and sensitive urinary assay is available to confirm the diagnosis of the kyphoscoliotic type of EDS. In this EDS subtype, deficiency of LH-1 results in a deficiency in hydroxylysine-based pyridinoline cross-links in types I and III collagen. As a result, cross-linked peptides are excreted in the urine as by-products of collagen turn-over. The diagnosis relies upon the demonstration of an increased ratio of lysylpyridinoline (LP) to hydroxylysylpyridinoline (HP) crosslinks in the urine measured by HPLC, which is a highly sensitive and specific test. In the kyphoscoliotic type of EDS, the LP/HP ratio is ~6, whereas normal ratios are ~0.2 [57]. In EDS spondylocheirodysplastic subtype, the LP/HP is slightly elevated to ~1 [42]. In the other EDS-subtypes, LP/HP ratio is are normal.

9.4.4 Molecular Genetic Testing

Molecular analyses usually start from genomic DNA (gDNA) and mRNA extracted from cultured dermal fibroblasts. For the classic subtype of EDS, the majority of mutations result in a non-functional *COL5A1* allele. As a first step therefore, a *COL5A1* "null-"allele test can be performed to determine if the individual is heterozygous for one of several *COL5A1* polymorphic exonic markers in the genomic DNA and to establish at the cDNA level whether or not both alleles are expressed. Sequence analysis of the genes involved in the various subtypes of EDS can be performed either on gDNA or cDNA. Once a mutation is identified in the patient, its presence or absence can easily be verified on gDNA obtained from other family members.

Prenatal diagnosis in at risk pregnancies is possible by analysis of DNA extracted from fetal cells obtained by amniocentesis usually performed at about 15–18 weeks' gestation or chorionic villus sampling (CVS) at about 10–12 weeks' gestation. The disease-causing allele of an affected family member must be identified before prenatal testing can be performed. Requests for prenatal testing for conditions like classic EDS which do not affect intellect or life span are not common. Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing, particularly if the testing is being considered for the purpose of pregnancy termination rather than early diagnosis. Although most centers would consider decisions about prenatal testing to be the choice of the parents, discussion of these issues is appropriate.

Preimplantation genetic diagnosis (PGD) may be available for families in which the disease-causing mutation has been identified in an affected family member.

9.5 Management

In view of the multisystemic involvement, different medical specialists need to be involved in the management of EDS patients, depending on the extent and severity of the disease manifestations. Each patient presents with a particular set of clinical problems, requiring a treatment plan that is tailored to his or her (specific) needs. Coordination of the multi-disciplinary approach to ensure that all areas of the syndrome are covered is preferably done by a clinical geneticist, who will also provide genetic counselling. No causal therapy is available for EDS; however, a series of 'preventive' guidelines are applicable to all forms of EDS. The key aspects of management include cardiovascular work-up, physiotherapy, pain management, and psychological follow-up.

A baseline echocardiogram should be performed in all patients with EDS in order to evaluate aortic root diameter, as adjusted for age and body surface area, and to evaluate cardiac valvular abnormalities. Because longitudinal data on

progression of aortic dilation are not available, specific recommendations for follow-up in individuals with a normal aortic diameter do not exist. However, if no abnormalities are found on echocardiogram in an adult, a follow-up echocardiogram is probably not necessary. For children and adolescents, it is reasonable to repeat the echocardiogram, approximately every 3 years until adulthood. Annual echocardiograms are warranted only if an abnormality such as aortic dilatation or mitral valve prolapse is present.

Children with pronounced skin fragility should wear protective pads or bandages over the forehead, knees and shins in order to avoid skin lacerations. Dermal wounds should be closed without tension, preferably in two layers. Deep stitches should be applied generously. Cutaneous stitches should be left in place twice as long as usual, and additional fixation of adjacent skin with adhesive tape can help to prevent stretching of the scar.

Patients with pronounced bruising are advised to avoid contact sports and heavy exercise. Protective pads and bandages can be useful in the prevention of bruises and haematomas. Drugs that interfere with platelet function and prolong the bleeding time should be avoided whenever possible. These include for example aspirin (acetylsalicylic acid, ASA), dipyridamole, clopidogrel, and non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and diclofenac. Paracetamol (acetaminophen) and COX-1 sparing NSAIDS, such as celecoxib, do not influence haemostasis and can be considered safe. By definition, anticoagulant drugs will increase the bleeding tendency, and should also be avoided, especially in the vascular subtype of EDS. Examples are the oral vitamin K antagonists (coumadins) such as acenocoumarol, fenprocoumon and warfarin, heparin and low molecular weight heparins (LMWH), and more recent oral thrombin inhibitors and pentasaccharides. Supplementation with ascorbic acid, a cofactor for cross-linking of collagen fibrils, can ameliorate the tendency towards bruising in some patients. The vasopressin analogue DDAVP (desmopressin acetate, 1-Desamino-8-D-Arginine Vasopressin) has been reported to reduce a bleeding tendency temporarily in subjects undergoing a dental or surgical procedure [14]. A case report describes the successful use of recombinant factor VIIa in a patient with the vascular type of EDS, in whom continued bleeding was successfully stopped after intravenous administration of recombinant factor VIIa [58].

In patients with hypotonia and delayed motor development, a physiotherapeutic program is important. Non-weight-bearing muscular exercise, such as swimming, is useful to promote muscular development and coordination. In contrast, sports with heavy joint strain, such as contact sports, should be discouraged. Besides prescription of physiotherapy, the rheumatologist and/or physical therapist may also provide assisting devices such as braces, ring splints, soft collar necks, while the occupational therapist may provide tools that make the living and working environment more comfortable for the patient. Many patients will be subjected to one or more orthopedic procedures prior to the diagnosis of EDS, such as arthroplasty and capsulorraphy, although with variable (or limited) success. The degree of joint stabilization, pain reduction, and duration of improvement is usually far less successful in patients with EDS than in those without this disorder. It is often preferable to delay surgery in EDS patients in favour of physical therapy and bracing.

Pain management is also very important. Pain medication should be tailored to the individual's subjective symptoms. Cognitive behavioural therapy can be beneficial in patients with hypermobility and chronic pain. Finally, psychological follow-up designed to explore coping strategies and to recognize and treat depression is of utmost importance.

For the vascular type of EDS, some prophylactic measures are of special interest.

Invasive vascular procedures such as arteriography and catheterization should be avoided because of the risk of vascular ruptures which cause significant morbidity and mortality [59, 60]. They should rather be replaced by ultrasonography and/or subtraction angiography. Surgical interventions are generally discouraged because of increased vascular fragility and a conservative approach is recommended in this condition.

When surgery is required for the treatment of arterial or bowel complications or other health problems, thorough investigation of platelet function and clotting is appropriate, as affected persons are already subject to bleeding from ruptured vessels or organs and an additional intrinsic clotting defect may complicate clinical outcome. Surgical exploration and intervention should be minimized and manipulation of vascular and other tissues should be done with extreme care [61]. Recently a multicentre randomised trial showed that celiprolol, a longacting β_1 antagonist with partial β_2-agonist properties, decreased the incidence of arterial rupture or dissection by three times in patients with the clinical diagnosis of vascular EDS. This study represents a substantial breakthrough in the evidence-based management of the syndrome [16].

Pregnancy for women with the vascular type of EDS is a high-risk venture. The risk of maternal death is as high as 12 % from uterine rupture or peripartum arterial rupture [62]. On the other hand, some women with vascular EDS have had one or several successful pregnancies, even prior to recognition of their underlying condition. It is prudent to follow pregnant women with the vascular type of EDS in a high-risk obstetrical program. It is not clear whether elective caesarean section is preferred to vaginal delivery.

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Ehlers–Danlos Syndrome Associated with Glycosaminoglycan Abnormalities

10

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Abstract

Ehlers–Danlos syndrome (EDS) is a genetically and clinically heterogeneous group of connective tissue disorders that typically present with skin hyperextensibility, joint hypermobility, and tissue fragility. The major cause of EDS appears to be impaired biosynthesis and enzymatic modification of collagen. In this chapter, we discuss two types of EDS that are associated with proteoglycan abnormalities: the progeroid type of EDS and dermatan 4-*O*-sulfotransferase 1 (D4ST1)-deficient EDS. The progeroid type of EDS is caused by mutations in *B4GALT7* or *B3GALT6*, both of which encode key enzymes that initiate glycosaminoglycan (GAG) synthesis. D4ST1-deficient EDS is caused by mutations in *CHST14*, which encodes an enzyme responsible for post-translational modification of GAG. The clinical and molecular characteristics of both types of EDS are described in this chapter.

Keywords

Ehlers–Danlos syndrome (EDS) • Progeroid type • *B4GALT7* • *B3GALT6* • Xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 • UDP-Gal:βGal β 1,3-galactosyltransferase polypeptide 6 • Dermatan 4-*O*-sulfotransferase 1 (D4ST1)-deficient EDS • *CHST14*

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Abbreviations

CHST14	Carbohydrate (*N*-Acetylgalactosamine 4-*O*) Sulfotransferase 14
D4ST1	Dermatan 4-*O*-sulfotransferase 1
EDS	Ehlers–Danlos Syndrome
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
GlcA	Glucuronic Acid
IdoA	Iduronic Acid
PG	Proteoglycan
Xyl	Xylose

10.1 Introduction

Ehlers–Danlos syndrome (EDS) is a heterogeneous connective tissue disorder that affects as many as 1 in 5,000 individuals. It is characterized by joint and skin laxity, and tissue fragility [44]. In a revised classification, Beighton et al. classified EDS into six major types and several minor types [2]. The major causes of EDS are thought to include abnormal collagen biosynthesis through dominant-negative effects, haploinsufficiency of mutant procollagen α-chains, or deficiencies in collagen processing enzymes [29]. Abnormal glycosaminoglycan (GAG) synthesis and incorrect post-translational modification of GAG in proteoglycans (PGs) were recently identified in the progeroid type of EDS (EDS, progeroid form; MIM#130070, MIM#615349) and dermatan 4-*O*-sulfotransferase 1 (D4ST1)-deficient EDS (EDS, musculocontractural type; MIM#601776), respectively. In this chapter, the clinical and molecular characteristics of both types of EDS are described.

10.2 Background

Glycosylation is the addition of a sugar chain (a glycan) to a protein (generating a glycoprotein) or lipid (generating a glycolipid). More than 40 human disorders are thought to be caused by abnormal glycosylation [15, 19]. PGs are composed of core proteins and one or more glycans with modifications. PGs are present in the extracellular matrix and have important diverse biological functions [5]. PG synthesis is initiated by the sequential addition of four monosaccharides (xylose [Xyl], two molecules of galactose [Gal] and glucuronic acid [GlcA]), known as a linker tetrasaccharide, to the serine residue of the core protein backbone (Fig. 10.1a). Additional sugar chains are extended from the linker tetrasaccharide by the addition of repeated disaccharides (usually consisting of 50–150 disaccharides in vivo). Afterwards, some sugars are modified by a series of epimerases (epimerization) and sulfotransferases (sulfation).

GAGs are long unbranched polysaccharides consisting of repeating disaccharide units. GAGs are highly negatively charged because of the acidic sugar residues and/or sulfation. Consequently, GAG can change its conformation, attract cations, and bind water. Hydrated GAG gels enable joints and tissues to absorb large pressure changes, providing tissue elasticity. Post-translational modifications such as epimerization, sulfation, and acetylation/deacetylation result in the formation of diverse motifs in the GAG chains, which can bind to a large variety of ligands. Therefore, GAG chains play important roles in regulating growth factor signaling, cell adhesion, proliferation, differentiation, and motility [3, 5, 45].

GAGs can be divided into two groups: (1) galactosaminoglycans such as chondroitin sulfate (CS) and dermatan sulfate (DS), and (2) glucosaminoglycans such as hyaluronic acid, keratan sulfate, heparan sulfate, and heparin [42]. Two types of glycosylation are known: *O*-glycosylation and *N*-glycosylation (Fig. 10.2a). Most GAGs (except for keratan sulfate and hyaluronic acid) are *O*-glycans that bind to the glycan via an oxygen molecule in the serine or threonine residue of the core protein (Fig. 10.2a). Notably, failure to add the first or second galactose residue of the tetrasaccharide results in the progeroid type of EDS (Fig. 10.1b, c).

The CS and DS GAGs are produced via the same pathway (Fig. 10.3a). In this pathway, after the linker tetrasaccharide attaches to the serine

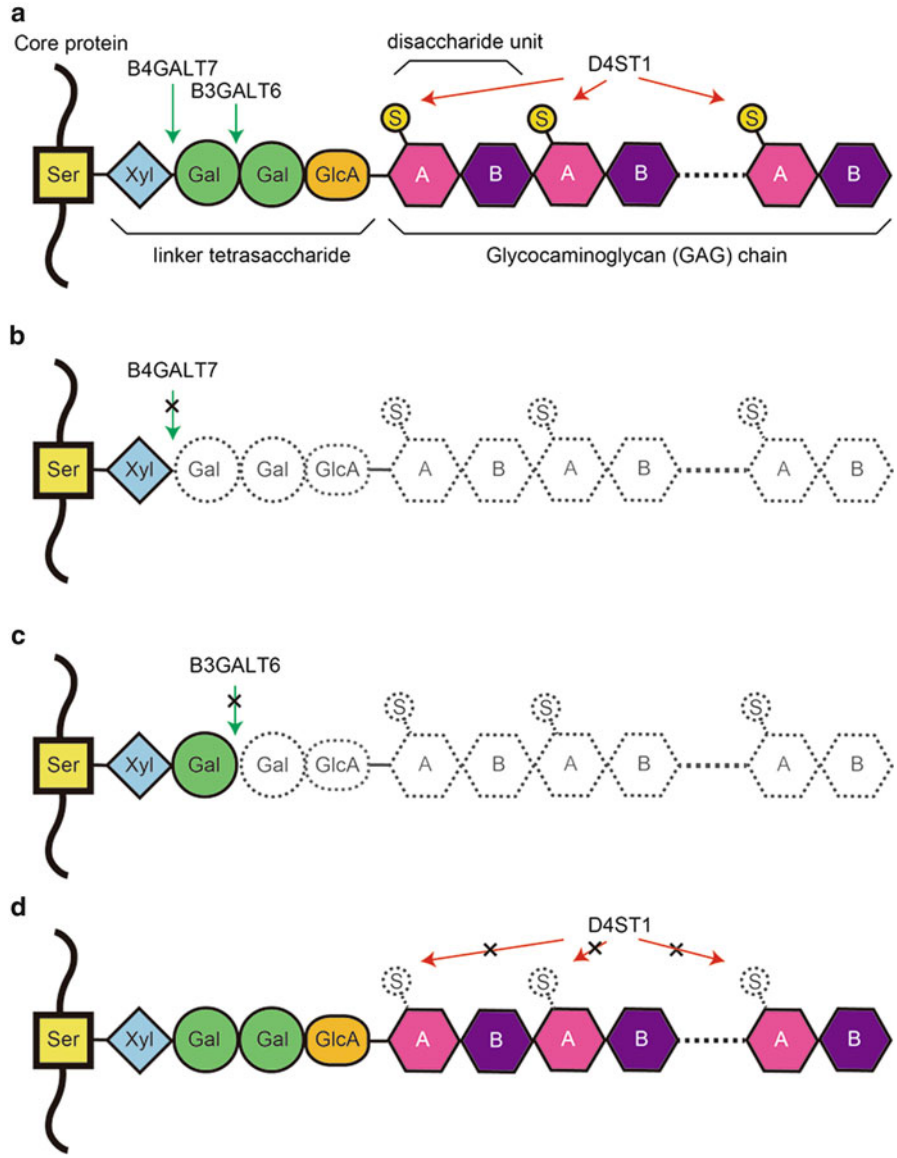

Fig. 10.1 Proteoglycan biosynthesis and its defects in two types of EDS. (**a**) Normal state. The serine residue (Ser) of the core protein and the GAG chain are bound via a linker tetrasaccharide. In CS, the disaccharides are composed of N-acetylgalactosamine (GalNAc) [position A] and glucuronic acid [position B]. In DS, the disaccharides are composed of GalNAc [position A] and Iduronic acid (IdoA) [position B]. B4GALT7 and B3GALT6 add the first and second galactose (Gal) to the xylose of the linker tetrasaccharide (*green arrows*). D4ST1 then adds the active sulfate to the 4-*O* position of GalNAc (*red arrows*) on DS. (**b**, **c**) Progeroid type of EDS. The impaired B4GALT7 cannot elongate the glycan chain from the first galactose (**b**). The impaired B3GALT6 cannot add the second galactose and the following glycan chain (**c**). (**d**) D4ST1-deficient EDS. The impaired/inactive D4ST1 cannot add the sulfate to GalNAc. *Gal* galactose, *GlcA* glucuronic acid, *S* active sulfate, *Ser* serine, *Xyl* xylose

residue of the core protein, GalNAc (*N*-acetyl galactosamine) transferase I elongates the glycan branch to create CS/DS. The enzyme C5-carboxy epimerase transforms glucuronic acid (GlcA) to iduronic acid (IdoA), which is specific for dermatan/DS (Fig. 10.3a). DS actually exists in a CS/DS hybrid state, containing GlcA–GalNAc and IdoA–GalNAc disaccharides (Figs. 10.2b

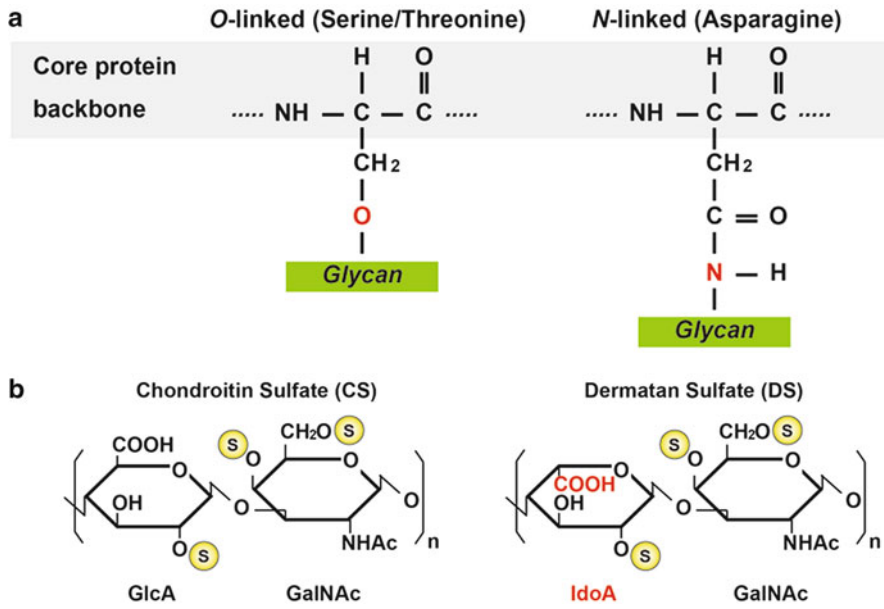

Fig. 10.2 Chemical structures of proteoglycan and disaccharides. (**a**) Chemical structure of *O*-linked and *N*-linked glycan. *O*-linked glycan can be linked via the *O*-element of serine or threonine. The diagram shows linking for serine. (**b**) Chemical structures of the disaccharide units of CS (*left*) and DS (*right*)

and 10.3a) [12]. Dermatan 4-*O*-sulfotransferase 1(D4ST1) specifically transfers an active sulfate to the 4-*O* position on the GalNAc residue of dermatan. The transfer of the active sulfate is impaired in D4ST1-deficient EDS (Figs. 10.1d and 10.3b).

10.3 The Progeroid Type of EDS (type 1: MIM#130070, type2: MIM#615349)

Alternative Names (MIM#130070)
Xylosylprotein 4-β-galactosyltransferase deficiency
XGPT deficiency
Galactosyltransferase I deficiency

10.3.1 Clinical Manifestations

Hernandez et al. reported five unrelated males in 1979, 1981, and 1986 representing a distinct variant of EDS. These males presented with a progeroid facial appearance, mild intellectual disability, and multiple nevi, in addition to hyperextensibility and fragility of skin, a high propensity for bruising, and joint hypermobility (particularly of the digits) [16–18]. A wrinkled face, curly and fine hair, scant eyebrows/eyelashes, telecanthus, periodontitis, multiple caries, low set/prominent ears, pectus excavatum, winged scapulae, and pes planus were observed in all five patients. Cryptorchidism and inguinal hernia were also noticed in four of the patients. Interestingly, the occurrence of the disorder in all of these patients was sporadic and the ages of their fathers were relatively advanced (33–55 years old). These characteristics prompted Hernandez et al. to speculate that the syndrome is caused by a de novo mutation [16].

In 1987, Kresse et al. reported a Danish male patient who was born to non-consanguineous healthy parents [26]. This patient presented with the clinical features observed in the original five patients, as well as a triangular head with a tiny face, frontal bossing, mid-face hypoplasia, a broad nasal bridge, prominent deep-set eyes, a small mouth, dental anomalies, low-set ears,

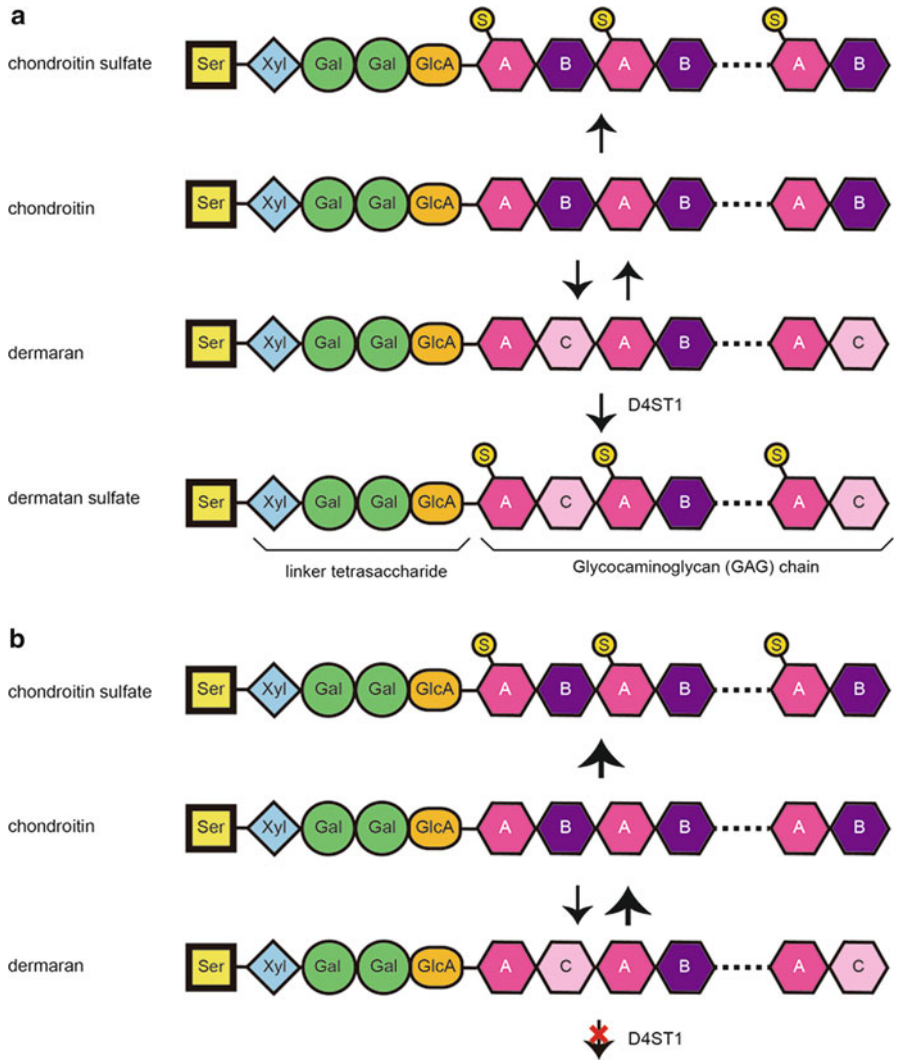

Fig. 10.3 Effects of D4ST1 defects on the biosynthesis of CS and DS. (**a**) The starting structure is chondroitin with a repeating disaccharide consisting of GalNAc [position A] and GlcA [position B]. Sulfation by 6-*O*-GalNAc sulfotransferase and 4-*O*-GalNAc sulfotransferase creates CS from chondroitin. To produce DS, first, C5-carboxy epimerase replaces GlcA with IdoA [position C]. This process is bidirectional as indicated by the bi-directional arrows. Then, D4ST1 adds sulfates to dermatan creating DS and prevents back epimerization. DS is often detected as a CS/DS hybrid. (**b**) In D4ST1-deficient EDS, back epimerization from IdoA to GlcA occurs. Consequently, neither DS nor dermatan are detected in fibroblasts derived from patients. *Gal galactose*, *GlcA* glucuronic acid, *S* active sulfate, *Ser* serine, *Xyl* xylose

short stature, osteopenia of all bones, dysplasia of some bones, and hypotonia. In 2004, Faiyaz-Ul-Haque et al. reported two patients from a large consanguineous Qatari family. The clinical features of both Qatari patients and the Danish patient seemed to be different from those of the original five patients [14].

10.3.2 Genetic Information

10.3.2.1 B4GALT7

In 1999, two different research groups [1, 33] identified compound heterozygous mutations of gene for xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7 (*B4GALT7*, NM_007255.2),

c.557C>A (p.Ala186Asp) and c.617T>C (p.Leu206Pro) in the Danish patient reported by Kresse in 1987 [26]. The two Qatari patients from a large consanguineous family were analyzed in 2004 [14]. Based on the hypothesis of autosomal recessive inheritance, haplotype analysis using microsatellite markers for the limited candidate loci delineated a homozygous region from *D5S469* and *D5S2111*, which harbors *B4GALT7* [14]. A homozygous missense mutation (c.808C>T, p.Arg270Cys) in *B4GALT7* was identified. Interestingly, the clinical phenotype of the Qatari patients was milder than that of the Danish one.

B4GALT7 was cloned by Okajima et al. [34]. The gene consists of six coding exons with a 948-bp open reading frame. This gene encodes xylosylprotein β-1,4-galactosyltransferase, polypeptide 7 (B4GALT7; aliases: galactosyltransferase I, XGPT1, and XGALT1), which is 327 amino acids long and its molecular weight is 37.4 kDa. B4GALT7 is a type II transmembrane protein localized in the Golgi apparatus, and is a key initiator of GAG synthesis as it attaches the first galactose of the linker tetrasaccharide of PGs (Fig. 10.1a, b).

10.3.2.2 B3GALT6

In 2013, Nakajima et al. have identified compound heterozygous mutations of *B3GALT6* (NM_080605.3) in three patients with progeroid form of EDS [32]. This intronless gene has a 990-bp open reading frame and encodes UDP-Gal:βGal β 1,3-galactosyltransferase polypeptide 6 (alternatively galactosyltransferase -II: GalT-II), which is 329 amino acids long and its molecular weight is 37.1kDa. It is also the type II transmembrane protein localized in the Golgi apparatus, and it attaches the second galactose of the tetrasaccharide linker of PGs (Fig. 10.1a, c). So far, two missense (c.16C>T, p.Arg6Trp and c.925T>A, p.Ser309Thr), two frameshift deletions (c.353delA, p.Asp118Alafs*160 and c.588delG, p.Arg197Alafs*81) and one in-frame deletion (c.415_423del, p.Met139Ala141del) were reported in this type of EDS [32].

10.3.3 Biochemical Characteristics

10.3.3.1 B4GALT7

Kresse et al. reported that their patient's fibroblasts produced only PG chain-free core proteins (molecular weight: 46 and 44 kDa) whereas control fibroblasts produced normal PG chains [26]. Additionally, the GAG-free core protein in that patient contained unsubstituted xylose residues (Fig. 10.1b).

Okajima et al. measured the enzyme activity of exogenously expressed proteins (wild type, p.Ala186Asp, p.Leu206Pro) in XGalT-1/B4GALT7-deficient CHO cells [33]. In total cell lysates, the enzyme activity of the p.Ala186Asp mutant was approximately 50 % lower than that of the wild-type protein, whereas the activity of the p.Leu206Pro mutant was almost undetectable. Interestingly, the wild-type and p.Ala186Asp proteins were localized in the Golgi apparatus whereas the p.Leu206Pro mutant existed in the cytoplasm. The α-helix disrupted by p.Leu206Pro may alter the protein's conformation, thus impairing intracellular trafficking and enzyme activity [33].

B4GALT7 activity in fibroblasts from another patient with a homozygous mutation, c.808C>T (p.Arg270Cys), was also lower than that of controls [40]. The extracellular matrix around the B4GALT7^{Arg270Cys} mutant fibroblasts was disorganized without banded fibrils. Furthermore, the proliferation of B4GALT7^{Arg270Cys} fibroblasts was significantly reduced to 45 % of the level of control fibroblasts [40].

Bui et al. measured galactosyltransferase activity of B4GALT7 mutants expressed in CHO pgsB-618 cells using 4-methylumbelliferyl-β-D-xylopyranoside as acceptor substrate. The enzyme activities of the p.Arg270Cys, p.Ala186Asp, and p.Leu206Pro mutants were decreased to 60, 11, and 0 % (undetectable) of that of the wild-type enzyme [4]. It has been reported that the clinical features of patients with the homozygous p.Arg270Cys mutation appear to be milder than those of patients with compound heterozygous mutations, including p.Ala186Asp or p.Leu206Pro, supporting the different effects of these mutations.

10.3.3.2 B3GALT6

Nakajima et al. measured the galactosyltransferase activity of *B3GALT6* in vitro using soluble-FLAG-tagged proteins for wild-type and mutant (p.Ser309Thr) which was observed common in two families and revealed the enzyme activity of the mutant protein was significantly decreased compared to the wild-type [32].

10.4 D4ST1-Deficient EDS (MIM#601776)

Alternative Names
Ehlers–Danlos syndrome, type VIB, formerly
Ehlers–Danlos syndrome, Kosho type
Ehlers–Danlos syndrome, musculocontractural type
Adducted thumbs, clubfoot, and progressive joints and skin laxity syndrome
Adducted thumb-clubfoot syndrome (ATCS)
Dündar syndrome
Arthrogryposis, distal, with peculiar faces and hydronephrosis

10.4.1 Clinical Manifestations

The kyphoscoliosis type of EDS (formerly known as, EDS type VI) is characterized by generalized joint laxity, severe muscular hypotonia and scoliosis at birth, scleral fragility, and rupture of the ocular globe [2]. This disorder is essentially caused by lysyl hydroxylase deficiency (EDS type VIA); other patients with similar clinical manifestations but without lysyl hydroxylase deficiency were classified as EDS type VIB.

In 2005, Kosho et al. reported two unrelated patients with fragile and hyperextensible skin, a high propensity for bruising, generalized joint laxity, kyphoscoliosis, and the major features of EDS VI, as well as a characteristic craniofacial appearance, and multiple congenital contractures [25]. Lysyl hydroxylase deficiency was excluded in these patients by analysis of the urinary deoxypyridinoline:pyridinoline ratio, and the patients were tentatively classified as EDS VIB. Kosho et al. subsequently reported on four additional unrelated patients and concluded that the patients represented a new type of EDS [23]. Notably, all six patients had homozygous or compound heterozygous mutations in *CHST14* [31]. Loss-of-function mutations in *CHST14* were independently found in 11 patients from four families with a rare arthrogryposis syndrome known as "adducted thumb-clubfoot syndrome (ATCS)" [9–11, 21, 43] and in three patients from two families who were originally classified as suffering from EDS VIB [27]. Malfait et al. suggested that these patients had the same disorder, which they termed "musculocontractural EDS" [27]. Shimizu et al. described the clinical characteristics of two additional patients together with a review of all of the patients reported at that time; their findings support the notion that the three independently identified conditions represent a single type of EDS [41]. Conversely, Janecke et al. claimed that the disorder should not be categorized as a type of EDS because of the presence of atypical clinical features, including facial dysmorphism, multiple congenital contractures, visceral anomalies, and impaired biosynthesis of DS as a cause of the disorder, and proposed the term DS-deficient adducted thumb-clubfoot syndrome [20]. In their response, Kosho et al. provided clinical and etiological evidence from which the disorder could be categorized as a type of EDS, because of the presence of all major features of EDS, including connective tissue fragility which required special and appropriate management of these patients. Decorin-mediated impaired assembly of collagen fibrils was the primary cause of progressive connective tissue fragility in this type [24]. Therefore, Kosho et al. proposed that the term D4ST1-deficient EDS (adducted thumb-clubfoot syndrome) was appropriate for this syndrome [24]. The current OMIM (http://www.ncbi.nlm.nih.gov/omim) registration of this disorder is EDS, musculocontractural type.

To date, descriptions of 26 patients (12 males, 14 females) from 17 families have been published [9–11, 21, 23, 25, 27, 30, 31, 41, 43, 46,

Table 10.1 Classification of Ehlers-Danlos syndrome

	Prevalence/patient number	Inheritance	Causative gene
Major types			
Classical type	1/20,000	AD	*COL5A1*, *COL5A2*
Hypermobility type	1/5,000–20,000	AD	Unknown[a]
Vascular type	1/50,000–250,000	AD	*COL3A1*
Kyphoscoliosis type	1/100,000	AR	*PLOD1*
Arthrochalasia type	30	AD	*COL1A1*, *COL1A2*
Dermatosparaxis type	8	AR	*ADAMTS2*
Other types			
Brittle cornea syndrome	11	AR	*ZNF469*
EDS-like syndrome due to tenascin-XB deficiency	10	AR	*TNXB*
Progeroid form	7	AR	*B4GALT7, B3GALT6*
Cardiac valvular form	4	AR	*COL1A2*
EDS-like spondylocheirodysplasia	8	AR	*SLC39A13*
D4ST1-deficient EDS (DD-EDS)	22	AR	*CHST14*

AD, autosomal dominant; *AR*, autosomal recessive; *COL5A1*, collagen, type V, alpha 1; *COL5A2*, collagen, type V, alpha 2; *COL3A1*, collagen, type III, alpha 1; *PLOD1*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; *COL1A1*, collagen, type I, alpha 1; *COL1A2*, collagen, type I, alpha 2; *ADAMTS2*, ADAM metallopeptidase with thrombospondin type 1 motif, 2; *ZNF469*, zinc finger protein 469; *TNXB*, tenascin XB; *B4GALT7*, xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7; *B3GALT6*, UDP-Gal:βGal β 1,3-galactosyltransferase polypeptide 6; *COL1A2*, collagen, type I, alpha 2; *SLC39A13*, solute carrier family 39 (zinc transporter), member 13; *CHST14*, carbohydrate (*N*-acetylgalactosamine 4-*0*) sulfotransferase 14

[a] *TNXB* mutations in a small subset of patients

48, 49]. This syndrome is characterized by a unique set of clinical features consisting of progressive systemic manifestations, including tissue fragility (e.g., skin hyperextensibility and fragility, progressive spinal and foot deformities, and large subcutaneous hematomas) and various malformations (e.g., facial features, congenital eye/heart/gastrointestinal defects, congenital multiple contractures). We have summarized the main clinical features of this syndrome in each organ in Table 10.1

10.4.1.1 Craniofacial Features

The characteristic craniofacial features apparent at birth or during early infancy include a large fontanelle, hypertelorism, short and down slanting palpebral fissures, blue sclerae, a short nose with hypoplastic columella, low-set and rotated ears, a high or cleft palate, a long philtrum, a thin upper-lip vermilion, a small mouth, and micro-retrognathia (Fig. 10.4a, b). Slender and asymmetrical facial shapes with a protruding jaw are generally observed from school age onwards (Fig. 10.4c, d).

10.4.1.2 Skeletal Features

Congenital multiple contractures, particularly adduction-flexion contractures of the thumbs and talipes equinovarus, are the main skeletal features (Fig. 10.4e, g, h). Fingers with a "tapering", "slender", and "cylindrical" shape are also common (Fig. 10.4f). Aberrant finger movement was described in three patients. Four patients had tendon abnormalities, including anomalous insertion of the flexor muscles, which probably caused the congenital contractures. Spinal deformities (e.g., scoliosis and kyphoscoliosis) and talipes deformities (e.g., planus and valgus) (Fig. 10.4i) occurred and progressed during childhood. Marfanoid habitus, recurrent joint dislocations, and pectus deformities (e.g., flat and thin, excavatum, and carinatum) were also evident. Bone mineral density was decreased in five patients and normal in two. Urine concentrations of the N-telopeptide of collagen type I, an osteoclast marker, were increased in three patients, whereas serum bone-specific alkaline phosphatase concentrations, an osteoblast marker, were normal in three, suggesting that

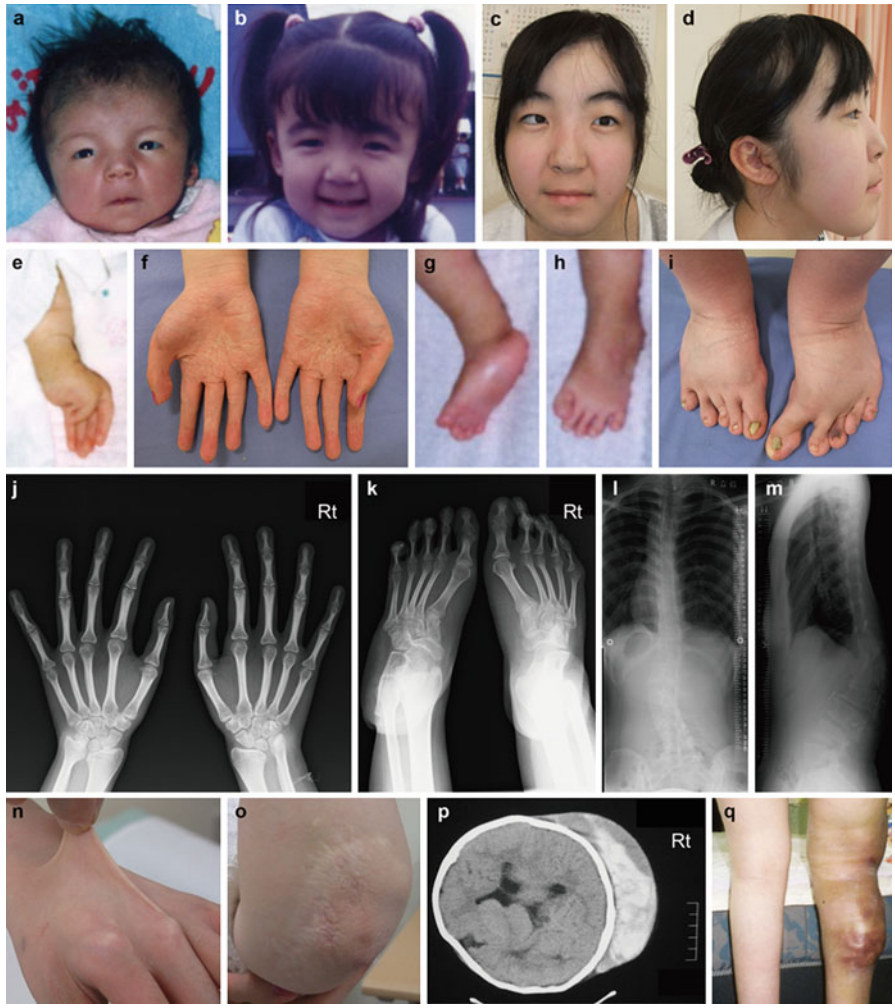

Fig. 10.4 Clinical photographs of patients with D4ST1-deficient EDS. (**a–d**) Facial features of a patient at 23 days (**a**), 3 years (**b**), and 16 years (**c**, **d**) of age. (**e**, **f**) Images of the hand in a patient with an adducted thumb at 1 month of age (**e**) and cylindrical fingers at 19 years of age (**f**). (**g–i**) Images of the foot in a patient with bilateral clubfeet at 1 month of age (**g**, **h**) and progressive talipes deformities (planus and valgus) at 19 years of age (**i**). (**j–m**) Radiographs of a 16-year-old patient show diaphyseal narrowing of the phalanges and metacarpals (**j**, **k**) and kyphoscoliosis with tall vertebral bodies (**l**, **m**). (**n**, **o**) Cutaneous features of a 19-year-old patient with hyperextensibility (**n**), atrophic scars, and fistula formation (**o**). (**p**) A massive cranial subcutaneous hematoma in the head of a 6-year-old patient after falling onto the floor. (**q**) A subcutaneous hematoma in the leg of a 16-year-old patient (All figures were originally published in Kosho et al. [23] except Fig. 10.4p, which was published in Kosho et al. [25])

increased osteoclast activity but normal osteoblast activity could cause osteopenia or osteoporosis. Radiologically, diaphyseal narrowing of the phalanges and metacarpals was noted in six patients (Fig. 10.4j, k). Talipes valgus and planus or cavum, with diaphyseal narrowing of the phalanges and metatarsals, were noted in six patients. Tall vertebral bodies were noted in five patients (Fig. 10.4l, m).

10.4.1.3 Cutaneous Features

Cutaneous features were apparent in most patients, including hyperextensibility to redundancy (Fig. 10.4n), a high propensity for bruising,

fragility leading to atrophic scars (Fig. 10.4o), acrogeria-like fine palmar creases or wrinkles, hyperalgesia to pressure, and recurrent subcutaneous infections with fistula formation. The palmar creases increased and became deeper with age.

10.4.1.4 Cardiovascular Features

Large subcutaneous hematomas were common, and frequently required intensive treatment, including hospital admission, blood transfusion, and surgical drainage (Fig. 10.4p, q). The lesions were thought to be caused by the rupture of a subcutaneous artery or vein. Bleeding time was prolonged in two patients (9 min and 11 min) and was normal in three. Intranasal administration of 1-desamino-8-D-arginine vasopressin prevented the development of large subcutaneous hematomas after trauma [49]. Four patients had congenital heart defects including an atrial septal defect in three, a patent ductus arteriosus in one, and coarctation of the aorta in one. Five patients had cardiac valve abnormalities including one who underwent surgery for infectious endocarditis, which was probably caused by aortic valve or mitral valve regurgitation.

10.4.1.5 Respiratory Features

Three adult patients developed pneumothorax or hemopneumothorax requiring chest tube drainage.

10.4.1.6 Gastrointestinal Features

Numerous gastrointestinal abnormalities were reported, including diverticular perforation in two adult patients, constipation in seven patients, abdominal pain in two patients, and other disorders in one patient (common mesentery, absence of the gastrocolic omentum with a spontaneous volvulus of small intestine, gastric ulcer, and malrotation with duodenal obstruction).

10.4.1.7 Genitourinary Features

Urological complications included nephrolithiasis or cystolithiasis in five patients, hydronephrosis in three, a dilated or atonic bladder with recurrent urinary tract infection in two, and a horseshoe kidney in one. Cryptorchidism was observed in eight male patients, including one who underwent orchiopexy because of hypogonadism in adulthood. Poor breast development was noted in five adolescent or adult patients. No pregnant females have been reported.

10.4.1.8 Ophthalmologic Features

Various ophthalmological complications have been reported, including strabismus in 12 patients, refractive errors in nine, glaucoma or elevated intraocular pressure in six, microcornea or microphthalmia in three, and retinal detachment in three.

10.4.1.9 Hearing Impairment

Six patients had hearing impairments, including for high-pitched sounds in three.

10.4.1.10 Growth

Patients showed mild prenatal growth retardation as the mean birth length was −0.5 standard deviations (SD), the mean birth weight was −0.6 SD, and the mean birth occipitofrontal circumference (OFC) was −0.2 SD. Postnatal growth was also mildly impaired, as the patients were generally slender with relative macrocephaly. The mean height was −0.9 SD, the mean weight was −1.5 SD, and the mean OFC was −0.2 SD.

10.4.1.11 Development and Neuromuscular Features

Gross motor developmental delay was observed in 14 patients, as the median age of independent walking was 2 years 1 month. Two patients, aged 15 years and 32 years, could not walk unassisted. An underlying myopathic process was observed in two patients. Mild intellectual disability was apparent in four patients. One patient had a global psychomotor delay at 1.5 years of age, but his intellectual quotient was approximately 90 at the age of 7 years 2 months. Brain imaging showed ventricular enlargement and/or asymmetry in seven patients, absence of the left septum pellucidum in one patient, and a short corpus callosum, mildly prominent Sylvian fissures, and periventricular nodular heterotopias. Two patients had spinal cord tethering.

10.4.2 Genetic Information

Autosomal recessive inheritance was considered based on the presence of this syndrome in consanguineous families [11, 27, 31]. Three independent groups have performed homozygosity mapping and/or linkage analysis and each showed that the gene carbohydrate (*N*-acetylgalactosamine 4-*O*) sulfotransferase 14 (*CHST14*, NM_130468.3) was responsible for this syndrome [11, 27, 31].

The *CHST14* gene was first cloned by Evers et al. [13]. It contains one coding exon (1,131-bp open reading frame) and is localized at 15q15.1. This gene encodes D4ST1, a 376 amino acid type II transmembrane protein (molecular weight: 43 kDa), that is localized in the Golgi membrane. It transfers a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate to position 4 of the GalNAc residues in dermatan to generate DS (Figs. 10.1a and 10.3a). Northern blotting revealed that *CHST14* is mainly expressed in heart, placenta, liver, and pancreas, and is weakly expressed in lung, skeletal muscle, and kidney [13].

To date, 11 pathogenic mutations of *CHST14* have been identified: p.Val49*, p.Lys69*, p.Arg135_Leu137delinsGlyThrGln, p.Phe209Ser, p.Arg213Pro, p.Lys226Alafs*16, p.Arg274Pro, p.Pro281Leu, p.Cys289Ser, p.Tyr293Cys, and p.Glu334Glyfs*107 [11, 27, 30, 31, 46, 48]. (p.Val48* was corrected to p.Val49*; Erratum in Am J Med Genet Part A 161A(2):403 (2013)) (p.Arg135Gly and p.Leu137Gln were originally reported by Dündar et al., but lately registered as c.403_410delCGCACCCTinsGGCACCCA, p.Arg135_Leu137delinsGlyThrGln in The Human Gene Mutation Database: https://portal.biobase-international.com/hgmd/pro/genesearch.php). Because these are protein truncation mutations and missense mutations, it seems likely that the mutations cause a loss of function.

10.4.3 Biochemical Information

Dündar et al. reported that DS-derived IdoA-GalNAc(4S) disaccharide was undetectable in fibroblasts derived from a patient with a homozygous p.Arg213Pro mutation. They also reported that GlcA-GalNAc(4S) content was greatly increased in the fibroblast extract and the culture media obtained from cultures of fibroblasts derived from this patient as compared with control fibroblasts [11]. It was also found that the amount of nonsulfated disaccharides (GlcA-GalNAc and IdoA-GalNAc) was increased in the cell extract and its media from the patient's fibroblast as compared with normal control fibroblasts. From these results, Dündar et al. proposed that epimerization of GlcA to IdoA by C5-carboxy epimerase is followed by sulfation of the C4 hydroxyl on the adjacent GalNAc residue by D4ST1. This process generates DS from dermatan and prevents back-epimerization from IdoA to GlcA [11, 28].

Miyake et al. measured the sulfotransferase activity of COS7 cells transfected with wild-type and mutant D4ST1 harboring the p.Lys69*, p.Pro281Leu, p.Cys289Ser, or p.Tyr293Cys mutations. The enzyme activity of the mutants was as low as that in mock transfected cells, suggesting that these missense mutations result in the loss of function [31]. The disaccharide composition of the decorin GAG chain isolated from the patient's fibroblasts consisted only of CS, without DS, while the chains isolated from normal fibroblasts consisted of CS/DS hybrid chains [31]. Furthermore, the level of nonsulfated dermatan was negligible in the patient's fibroblasts [31]. Thus, in this syndrome, the CS/DS chain is replaced with the CS chain, even though the core proteins are normal.

10.4.4 Pathology and Pathophysiology

Of the major DS proteoglycans in skin, decorin was a focus of research because it binds to collagen fibrils via its core protein and its GAG chains act as interfibrillar bridges [38, 39]. Three α collagen chains are self-assembled to generate tropocollagen, in the form of a triple helix. Tropocollagen then self-assembles to form collagen fibrils via decorin (Fig. 10.5a). Collagen fibrils are assembled into a collagen fiber, known as the collagen bundle, via the antiparallel com-

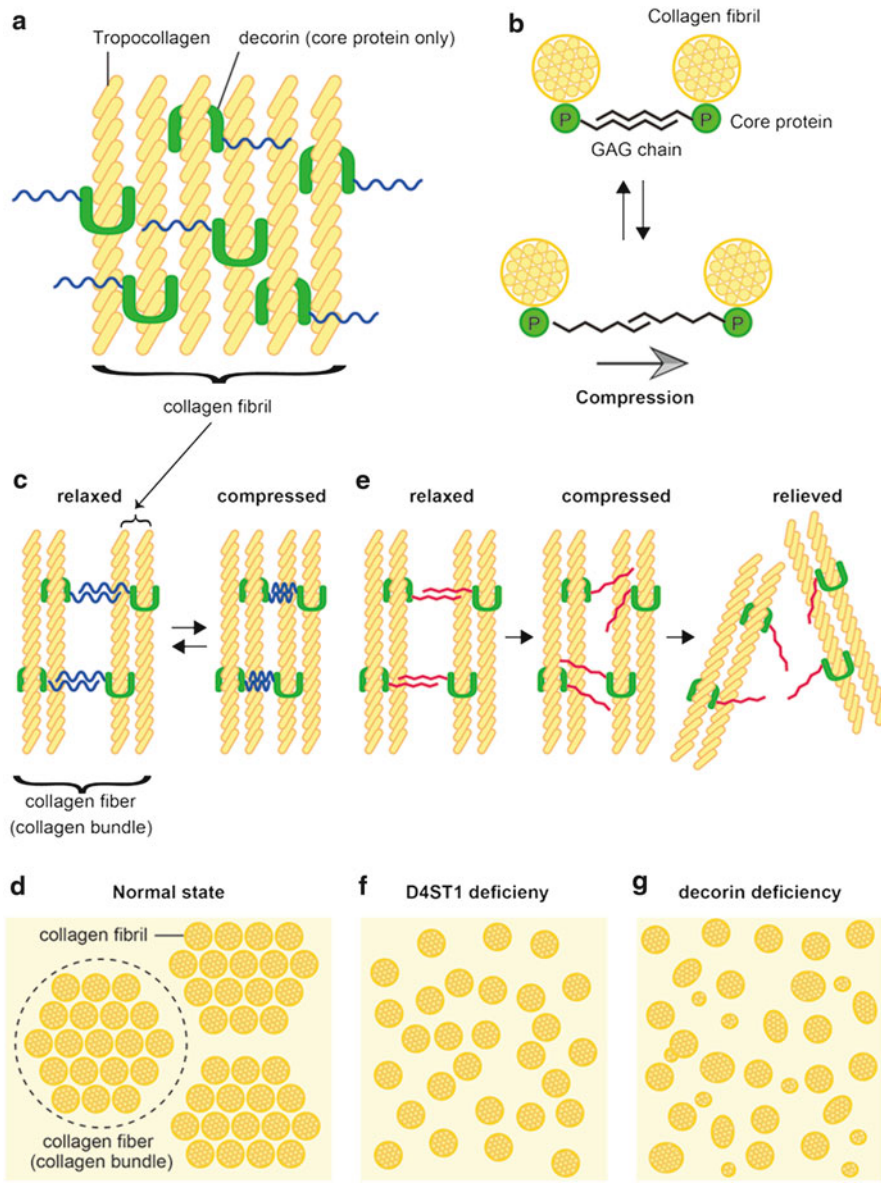

Fig. 10.5 Putative model of abnormal collagen bundle assembly in D4ST1-deficient EDS. (**a**) Tropocollagen directly binds to decorin and forms a collagen fibril. The *blue lines* represent the CS/DS hybrid chain. (**b**) Illustration of the sliding filament model showing reversible longitudinal slippage between the antiparallel GAG chains. The *black lines* represent unspecified GAGs. (**c**, **d**) In the normal state, the CS/DS chains can bend against the direction of mechanical compression and rebound to the original structure (**c**). Thus, the collagen bundles are refractory to compression stress (**d**). (**e**, **f**) In D4ST1-deficient EDS, the CS/DS chains are replaced with CS chains (*red lines*). These chains cannot resist mechanical compression, resulting in irreversible scattering of the collagen fibrils. (**g**) The size and shape of the collagen fibrils are highly variable in decorin-deficient mice

plex of the CS/DS hybrid GAG chains of decorin, which acts like a bridge to provide a space between individual fibrils and tighten the collagen fiber (Fig. 10.5c, d).

The GAGs span collagen fibrils in the extracellular matrix of skin and tendons, and the length of the GAG chain determines the width of the interfibrillar gap [35, 36]. Elasticity of the

extracellular matrix is explained by the sliding filament model, which allows reversible longitudinal slippage between the antiparallel GAG chains (Fig. 10.5b) [39]. Because tissue stability and elasticity depend on the structure of the GAG bridges, irreversible damage can occur if the bridges are inelastic [39].

Decorin is composed of a horseshoe-shaped core protein (molecular weight: ~45 kDa) and a single CS/DS hybrid chain on the N-terminal side (Fig. 10.5a) [22, 47]. Weber et al. reported that the model structure of decorin consists of an arch in which the inner concave surface is formed from a curved β-sheet and the outer convex surface is formed from α-helices. They also proposed that one tropocollagen fiber lies within the decorin convex and another interacts with one arm of the arch [47]. The IdoA:GlcA ratio in DS ranges from ~10 to >90 % depending on the tissue type [39]. Importantly, L-IdoA residues in DS can easily undergo conformational changes, unlike GlcA in CS [6, 7]. Thus, the IdoA:GlcA ratio should be higher in more flexible tissues [39].

Light microscopic investigation of skin specimens from two patients showed that fine collagen fibers were predominant in the reticular to papillary dermis and the number of thick collagen bundles was markedly reduced [31]. Electron microscopic examination of the specimens showed that collagen fibrils were dispersed throughout the reticular dermis, whereas they were regularly and tightly assembled in control tissue. Surprisingly, each collagen fibril was smooth and round, with little variation in size or shape, similar to the fibril in the control tissue (Fig. 10.5d, f) [31]. The disaccharide composition of the decorin GAG chain from a patient's fibroblasts only consisted of CS, without DS disaccharide, whereas control fibroblasts consisted of a CS/DS hybrid [31]. The transition of decorin from the CS/DS hybrid chain to a CS chain probably decreases the flexibility of the GAG chain. The sliding filament model proposes that mechanical compression might also work in the CS chain of D4ST1-deficient patients, but the inflexibility of the CS chain is unable to tolerate higher mechanical pressures or is too inelastic to maintain normal skin properties (Fig. 10.5e, f). This irreversible event could explain the progressive clinical course of this disease.

Interestingly, there were marked variations in the size and shape of dermal collagen fibrils in decorin-null mice (Fig. 10.5g) [8]. These findings suggest that the decorin core protein is important for collagen fibril formation, and that the CS/DS hybrid chain of decorin PG regulates the space between the collagen fibrils and form collagen bundles, as previously reported [37]. These findings suggest that the main pathological basis of this disorder could be insufficient assembly of collagen fibrils.

However, Dündar et al. reported that the light microscopic and electron microscopic findings of a patient's skin were unchanged compared to the normal control [11]. Malfait et al. reported that, in their patient, most collagen bundles were small diameter in size, and some were composed of collagen fibrils of varying diameter that were separated by irregular interfibrillar spaces [27]. In addition, the fibroblasts exhibited an elongated and/or dilated endoplasmic reticulum. So far, definitive histopathologic characteristics have not been established, so further studies are strongly encouraged to determine the major histological characteristics and underlying pathophysiology of this disorder.

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Cutis Laxa

11

Miski Mohamed, Michiel Voet, Thatjana Gardeitchik, and Eva Morava

Abstract

Cutis laxa is an inherited or acquired disease characterized by redundant, sagging and inelastic skin. In inherited cutis laxa an abnormal synthesis of extracellular matrix proteins occurs due to genetic defects coding for diverse extracellular matrix components. Recently, different inborn errors of metabolism have been found to be associated with cutis laxa as well. In some of these metabolic conditions the pathomechanism of cutis laxa remains unknown. Cutis laxa can be inherited in an autosomal dominant, autosomal recessive and X-linked recessive inheritance pattern. Besides the skin abnormalities, in most inherited forms multiple organ systems are involved, leading to a severe, in some forms even lethal, multisystem disorder. To date no effective treatment is available for cutis laxa. This chapter focuses on inherited forms of cutis laxa, offering a practical guideline for clinicians, biochemist and geneticist to diagnose and differentiate between the different forms of cutis laxa, and providing a concise theoretical reference.

Keywords

Autosomal recessive cutis laxa (ARCL) • ARCL type 1A (ARCL1A; MIM 219100) • *FBLN5* (MIM 604580) • ARCL type 1B (ARCL1B; MIM 614437) • EFEMP2 or *FBLN4* (MIM 604633) • ARCL1C • Urban-Rifkin-Davis Syndrome (URDS; MIM 613177) • *LTBP4* (MIM 604710) • ARCL type 2 • Debré-type cutis laxa (ARCL2A; MIM 219200)

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11.1 Introduction

Cutis laxa is a disease characterized by wrinkled, redundant, inelastic and sagging skin (Fig. 11.1), caused by defective elastin synthesis or structural abnormalities of the extracellular matrix [1]. The disease can be acquired or inherited. The inherited form of cutis laxa has an incidence of 1–2:400,000 [2]. The inborn types of cutis laxa show different inheritance patterns including autosomal dominant (ADCL; MIM 123700), autosomal recessive (ARCL; MIM 219100, MIM 219200, MIM 219150, MIM 231070) and X-linked recessive inheritance (XRCL; MIM 304150, Fig. 11.1) [3–5]. Within these patterns of inheritance, further distinction can be made between specific genetic defects.

Some forms of inherited cutis laxa either because of their familial segregation (autosomal dominant form), or due to their syndromal appearance (e.g. those with autosomal recessive inheritance; Fig. 11.1a–c) are clinically recognizable with relative ease.

Patients with autosomal dominant inherited cutis laxa (ADCL; MIM123700) are mostly diagnosed in early childhood with a mild form of cutis laxa with some systemic involvement (hernias, cardiovascular manifestations, gastrointestinal diverticula and emphysema) [6–8]. Complications are less common in these patients and their life span is usually normal. Each of the two mutations

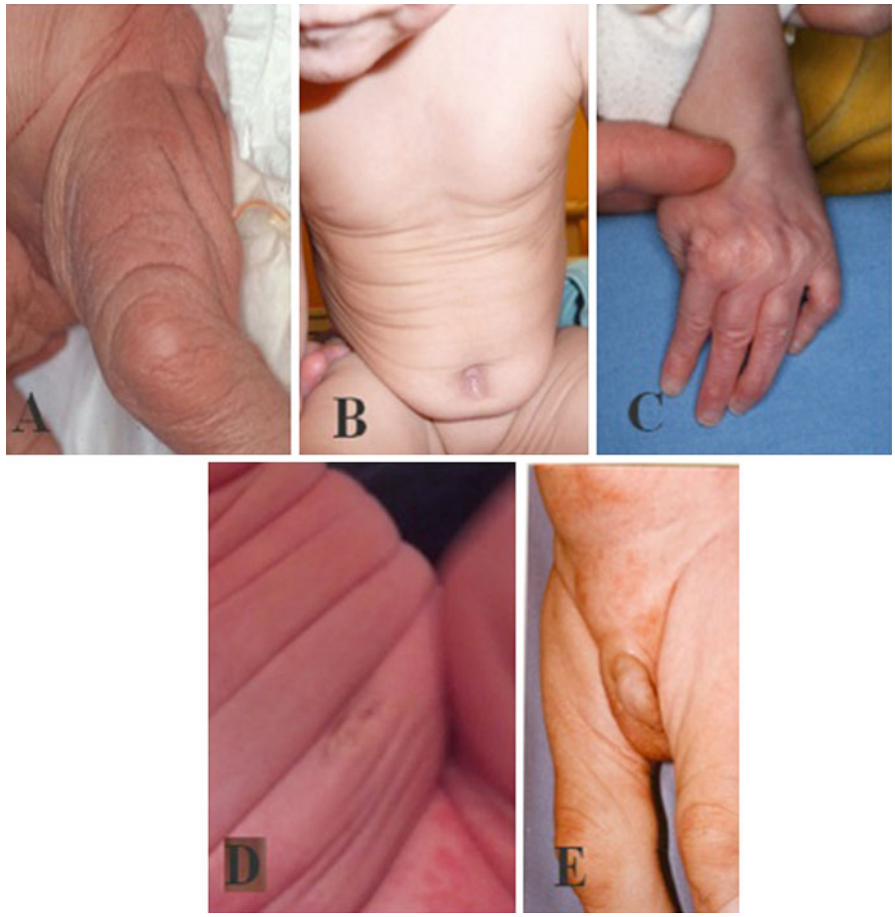

Fig. 11.1 Comparison of skin abnormalities in different subtypes of recessive cutis laxa syndromes. (**a**) Wrinkled skin and abnormal fat distribution in ARCL2A; (**b**) abdominal cutis laxa in ARCL2B; (**c**) parchment-like skin in ARCL3; (**d**) excess of loose skin in XLCL; (**e**) dry, wrinkled skin in GO

causing this disease, either in the elastin gene (*ELN*; MIM 130160) [9] or in the fibulin-5 gene (*FBLN5*; MIM 604580), can be inherited in an autosomal dominant manner [6, 10, 11].

X-linked recessive cutis laxa is caused by a mutation in the *ATP7A* gene (MIM 300011) leading to dysfunction of the ATP7A protein, a copper-transporting ATPase expressed in almost all tissues except the liver [12–14]. This mutation can lead to either X-linked cutis laxa (Fig. 11.1d), also known as Occipital Horn Syndrome (OHS; MIM 304150) or Menkes disease (MD; MIM 309400). Menkes disease can be considered a more severe type of OHS. Patients with an X-linked cutis laxa syndrome have a distinct, unique presentation at birth. Besides generalized cutis laxa they have a thin face, long philtrum, hooked and beaked nose, brittle hair, high forehead and large fontanel, giving a distinct facial appearance. Systemic involvement includes failure to thrive due to chronic diarrhea, malabsorption, congenital hydronephrosis, urethral and bladder diverticulae. Skeletal abnormalities are common and the specific sign of occipital horn exostoses of the skull is diagnostic for OHS [15].

Autosomal recessive cutis laxa (ARCL), is a separate type of often lethal, generalized connective tissue disorder with severe systemic manifestations [1]. It can be divided into many subtypes. ARCL type 1A (ARCL1A; MIM 219100) is caused by a mutation in the fibulin-5 gene (*FBLN5*; MIM 604580), which causes a severe form of cutis laxa [11, 16–19]. ARCL type 1B (ARCL1B; MIM 614437) is caused by a mutation in the EGF-containing fibulin-like extracellular matrix protein 2 gene or fibulin-4 gene (EFEMP2 or *FBLN4*; MIM 604633) [20, 21]. Cutis laxa with severe pulmonary, gastrointestinal, and urinary abnormalities or Urban-Rifkin-Davis Syndrome (URDS; MIM 613177) [22], caused by a mutation in the latent transforming growth factor-beta-binding protein 4 (*LTBP4*, MIM 604710), can be considered ARCL1C. Cutis laxa caused by a mutation in the elastin gene (*ELN*; MIM 130160) might also be inherited in an autosomal recessive pattern, but this has only been described in three patients from two related consanguineous Syrian families [23].

Besides ARCL type 1, there is ARCL type 2, which is more benign and genetically heterogeneous [1, 24, 30]. ARCL type 2 can also be subdivided. ARCL type 2A, or Debré-type cutis laxa (ARCL2A; MIM 219200) is caused by mutations in the gene encoding for the H+ transporting a2 subunit of the vesicular ATPase complex (*ATP6V0A2*; MIM 611716). Mutations in this gene cause a distinct phenotype characterized by congenital cutis laxa, intrauterine growth retardation, failure to thrive, microcephaly, multiple dysmorphic features, and a large and late closing fontanel [25–27] (Fig. 11.1a). Additionally, hypotonia, feeding problems, ocular anomalies (strabismus and myopia), hyperlaxity of joints, congenital hip dislocations, decreased bone mineral density and an abnormal fat distribution might occur. Developmental delay is observed in the majority of patients (mainly due to muscle hypotonia and joint laxity) but brain anomalies are rare [28]. Neurological symptoms include psychomotor retardation, seizures, hearing loss and cobble-stone like brain dysgenesis (Fig. 11.2) [29, 30]. In this unique form of cutis laxa syndrome, the skin features seem to improve over time [31]. Mutations in the *ATP6V0A2* can also cause Wrinkly Skin Syndrome (WSS), with a more distinct skin involvement, characterized at birth by wrinkled skin of the hands and feet with an increased number of wrinkles on the palms and soles and less systemic involvement compared to the Debré-type [22, 29, 30, 32]. Much still remains unknown about this type of cutis laxa, for instance, how a dysfunction in this proton pump V-ATPase leads to severe brain developmental defects (cobble-stone like brain dysgenesis). Even more interesting is why the skin symptoms and clinical course of these patients seems to improve over time [33].

Another specific subtype of cutis laxa is ARCL type 2B (ARCL2B; MIM 612940), caused by a mutation in the pyrroline-5-carboxylate reductase 1 gene (*PYCR1*; MIM 179035) [34, 35]. This type of cutis laxa is associated with mild mental retardation, a typical facial appearance including triangular face, molar hypoplasia and dysplastic and large ears, osteopenia and a typical wrist position with contractures and an adducted

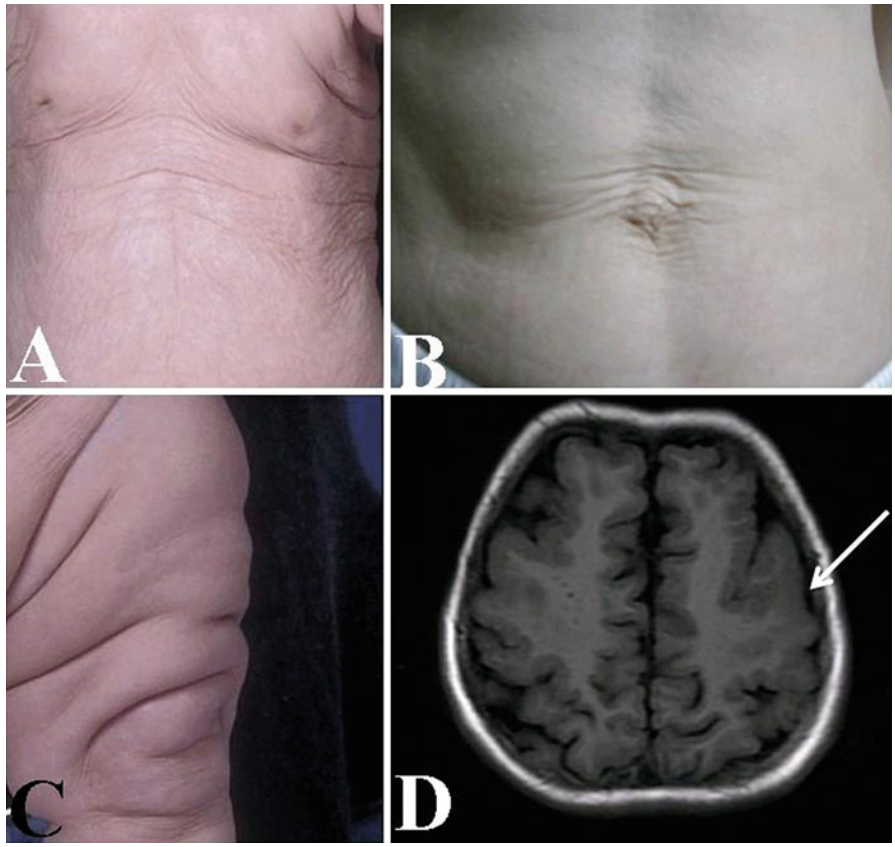

Fig. 11.2 Typical physical findings in ARCL2A. (**a**) Inverted nipples; (**b**) abdominal wrinkled skin; (**c**) abnormal fat distribution on the thighs; (**d**) cobbelstone-like brain dysgenesis

thumb [34]. Several patients carrying *PYCR1* mutations with ARCL2B have neurological symptoms, dystonia, spasticity and eye anomalies, including corneal opacity and cataract, which are known features of De Barsy syndrome, previously labeled as ARCL type 3B (ARCL3 B; MIM 614438) [34–38] (Fig. 11.1b).

De Barsy syndrome is actually one of the most intriguing forms of ARCL. It presents with several features overlapping with ARCL type 2, including skin symptoms, facial features, cranial abnormalities and growth delay. The disorder is frequently progressive, associated with dystonia, eye anomalies (cataract and corneal abnormalities) and progeroid features [35, 39, 40]. De Barsy Syndrome type A, previously known as ARCL type 3A (ARCL3 A; MIM 219150), is caused by a mutation in the aldehyde dehydrogenase 18 family, member A1 gene or Delta-1-pyrroline-5-carboxylate synthetase gene (*ALDH18A1* or *P5CS*; MIM 138250) [36] (Fig. 11.1c). De Barsy syndrome is genetically heterogenous, and also caused by mutations in *PYCR1*, and in a single case it has been associated with *ATP6V0A2* mutations [37].

Another recognizable type of ARCL type 2 is Gerodermia Osteodysplastica (GO; MIM 231070), which means "old skin" and "bone malformations", clearly describing the most striking clinical features (Fig. 11.1e). Spontaneous fractures and osteoporosis or severe osteopenia is present in the majority of patients. Interestingly,

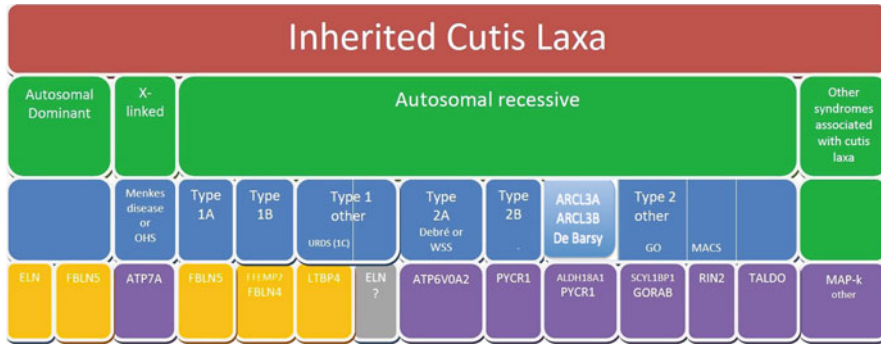

Fig. 11.3 Cutis laxa types and the underlying gene mutations. Note the novel nomenclature for De Barsy syndrome and genetic overlap between ARCL2B and ARCL3

bisphosphonate treatment seems to repair the bone anomalies and restore normal bone density in patients with GO [41]. Gerodermia Osteodysplastica is caused by a mutation in the Golgi, RAB6-interacting gene (*GORAB*, MIM 607983) and patients often have normal intelligence.

Another type of ARCL2 is "Macrocephaly, alopecia, cutis laxa, and scoliosis" (MACS; 613075), which is caused by a mutation in the RAS and RAB interactor 2 gene (*RIN2*; MIM 610222), also affecting the Golgi apparatus. This syndrome is recognizable due to sagging chin and prominent mouth and a progressive facial coarsening [42–44].

Cutis laxa in transaldolase (*TALDO*) deficiency (MIM 606003) patients was described for the first time in 2006 by Valayannopoulos et al. They reported a new sibship of four infants with consanguineous parents. All of them presented at birth or in the antenatal period with dysmorphic features, cutis laxa and hypertrichosis, hepatomegaly, splenomegaly, liver failure, hemolytic anemia, thrombocytopenia and genitourinary malformations. A mutation in the *TALDO1* gene was found in these patients [45]. As cutis laxa has not been described in other *TALDO1* patients before and the etiology of cutis laxa in these patients remained so far unknown, it was suggested that this finding was coincidental and due to the consanguinity in this family [33]. Based on a recent report on Saudi Arabic patients with *TALDO1* defect it seems that the majority of the patients have facial wrinkling and cutis laxa of the forehead.

Next to these inherited types of cutis laxa, there are many more syndromes or (metabolic) diseases associated with cutis laxa, e.g. and MAP kinase pathway defects [46]. Mitogen-activated protein (MAP) kinases comprise a family of ubiquitous proline-directed, protein-serine/threonine kinases, which participate in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms. MAP kinases function in protein kinase cascades [46].

The different types of inherited cutis laxa have distinct clinical and metabolic features (see above). The specificity of these clinical signs can help to target genetic diagnosis, which could be costly and time-consuming due to the high number of candidate genes. Some metabolic and imaging features (abnormal glycosylation and cobble stone like dysgenesis detected by brain imaging in ARCL 2A, parchment-like skin and joint luxations/contractures in ARCL 2B, occipital horns on X-ray in X-linked CL or abnormal wrist position with contractures in ARCL 2B) are so specific that they can even lead to direct genetic diagnosis (flow diagram, Fig. 11.3).

11.2 Autosomal Dominant Cutis Laxa Syndrome (ADCL, MIM 1301160)

ADCL has been described to occur due to *ELN*, and in a few cases due to *FBLN5* mutations [6, 47]. Some of the mutations occur de novo. This type of cutis laxa is not always present at birth, and might appear in late childhood or early adulthood in patients. In familial cases the family history shows variable penetrance and some of the affected individuals in the family may demonstrate only facial (sagging chin or sagging eye-lids) cutis laxa. In these cases, especially if the disorder shows a late onset, familiality could be missed. Associated features are variable, mostly including the cardiovascular system and eventual lung involvement. Inguinal hernias, bladder diverticula or vesico-urinary reflux are rare. Patients have normal intelligence.

Heterozygous loss-of-function *ELN* mutations cause alterations of tropoelastin expression and defected elastic fiber formation, affecting the vascular system and less severely the skin [6]. Next to generalized cutis laxa, sagging chins and a droopy look some patients with *ELN* mutations might also develop aortic aneurysms [9, 24]. This life threatening complication needs invasive and sometimes preventive surgery similar to the aneurysmal cystic media degeneration seen in Marfan syndrome (MIM 154700). Patients with *ELN* mutations might have a more severe phenotype if the disorder is caused by a chromosomal deletion on the long arm of chromosome 7, especially when it is present as part of a contiguous gene syndrome (Williams syndrome, MIM 194050). Williams syndrome is a common microdeletion syndrome with a complex phenotype of developmental delay, characteristic face, cardiovascular malformations, recognizable behavior and hypercalcemia, involving the *ELN* locus (Fig. 11.4).

Based on the description of the family reported with ADCL due to *FBLN5* mutations, and based on our own experience, this rare, dominant form seems to be milder in clinical presentation. Internal organ involvement might be absent. Intriguingly some of the patients described with heterozygous *FBLN5* mutations demonstrate age-related macular degeneration (ARMD 3, MIM 608895) and peripheral neuropathy. Abnormal fibulin 5 secretion has been demonstrated in several of these patients [17, 24]. Even more interestingly ARMD 3 has been also shown to be associated with mild cutis laxa in a subgroup of these *FBLN5* mutation carrier individuals. Macular degeneration, or peripheral neuropathy, however, has not been observed in ADCL patients yet [48–50].

Fibulins are microfibrillar proteins, making a net into which secreted tropoelastin is transported. Lysyl oxidases, a group of copper-dependent enzymes, are necessary for the conversion of tropoelastin into elastin to form crosslinks between molecules. Abnormal tropoelastin secretion and abnormal microfibrillar network production leads to the same consequence, a defective extracellular matrix structure, and cutis laxa. ADCL has similar skin abnormalities and a comparable organ involvement to that of ARCL1 [24]. The pathomechanism of fibuline 5 related autosomal dominant cutis laxa, however, is very different from that in ARCL1. The mutated protein in ADCL patients is changed in conformation, but it appears to be stable, and has a

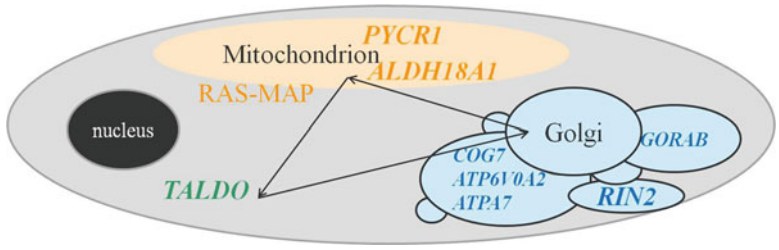

Fig. 11.4 Cellular localization of genes associated with cutis laxa and interconnections of the related metabolic pathways

dominant negative effect on the secretion and network forming of elastic fibers, leading to the classic cutis laxa phenotype.

11.3 Autosomal Recessive Cutis Laxa Type I (ARCL1)

Most ARCL1 patients have a severe presentation of a generalized connective tissue disorder presenting in early childhood in association with generalized cutis laxa. Inguinal and umbilical hernias, vesico-urinary and gastro-esophageal reflux and diverticula formation are present in some degree in all affected patients. Bladder and intestinal diverticula and/or pyloric stenosis with cutis laxa are diagnostic signs for ARCL1 [24]. Severe pulmonary emphysema is the most common, and discriminative finding for recessively inherited ARCL1. Pulmonary emphysema has been described in both *FBLN5*- (ARCL1B, MIM 219100) [16, 18, 19] and *FBLN4 or EFEMP2-related* (ARCL1A; MIM 219100) cutis laxa [20, 21, 24], and in the recently discovered LTBP4 related URDS (ARCL1C; MIM 613177). Cardiac involvement might be variable in ARCL-1, including peripheral pulmonary artery stenosis or supra-valvular aortic stenosis. Intra-familial variability has been observed in age of onset in ARCL1. Affected siblings have been reported but no clinical features were noted in the carrier parents. The joints are not involved in this specific autosomal recessive type, which is the second important feature after the presence of early pulmonary emphysema, distiguishing patients with ARCL1 from patients with ARCL2. Another characteristic feature is that intelligence is not affected in ARCL1. Hip dislocation is not observed in *FBLN5*-related cutis laxa.

The disease course of ARCL1 depends on the cardiac and pulmonary involvement [21]. Lung emphysema, recurrent pulmonary infections and cardiac failure due to congenital heart malformations determine the long term survival; most children die in early childhood or before puberty, and survival to adulthood is exceptional. The most common mutation in ARCL1A is the homozygous base change T998C, resulting in a severe chronic disease and lethality at the age of 6–14 years, with one case of exceptional long survival to the age of 21 years in an affected woman.

The pathomechanism of fibulin 5 and fibulin 4 related autosomal recessive cutis laxa is the defective synthesis of the complex network of the microfibrillar components of elastic fibers [21]. *FBLN5* mutations in ARCL are loss of function mutations, leading to abnormal protein folding and a delayed secretion of fibulin 5 to the extracellular matrix in affected individuals. In a peculiar case of recessive *ELN* mutations in association with a heterozygous p.Gly202Arg *FBLN5* mutation the patient had cutis laxa with progressive elastolysis [24].

Differential diagnosis between the different types of ARCL1 is rather difficult and relies on the presence or absence of gastrointestinal and urinary involvement (less gastrointestinal involvement in *FBLN4* mutations compared to *FBLN5* cases, urinary diverticuli in FBLN4 cases, and very severe involvement of both gastrointestinal and urinary systems in *LTBP4*). The other difference is the presence of vascular involvement in fibuline-related defects. *FBNL4-related* cutis laxa patients might also have arachnodactyly and arterial tortuosity with a predisposition for aneurysms and dissections, which is rare in the other two gene defects [24].

11.4 Metabolic Cutis Laxa Syndromes

To date, a growing number of metabolic disorders have been found to be associated with inherited cutis laxa. The first metabolic disease reported with old-looking, wrinkled skin was Menkes disease. Next to this disorder with an X-linked recessive inheritance, most of the metabolic cutis laxa syndromes are inherited in an autosomal recessive fashion. Surprisingly, cutis laxa has also been associated recently with abnormal glycosylation. The group of metabolic cutis laxa is rather heterogeneous and the genetic cause in many patients remains unsolved. Although the clinical and biochemical

features partially overlap, there are discriminative features characteristic for each specific metabolic cutis laxa disorder.

11.4.1 Menkes Disease

Menkes disease (MD; MIM 309400) first described in 1962, is a fatal X-linked recessive disorder characterized by progressive neurological dysfunction, growth retardation and kinky hair. The characteristic abnormality of the scalp hair is caused by broken and twisted hair (trichorrhexis nodosa and pili torti, respectively) and varying diameter of the hair shaft [51]. Besides these classical symptoms, MD patients show skeletal anomalies, vascular defects and hypothermia due to a generalized connective tissue disorder [52].

A defect in the *ATP7A* gene (MIM 300011), coding for the ATP7A protein, a copper-transporting ATPase, is responsible for causing MD [52, 53]. Expression profiles show ATP7A expression in almost all tissues with an exception of the liver [12–14]. Due to the defective protein, copper is entrapped in the intestinal mucosa and cannot be absorbed and transported into the different tissue, leading to a severe systemic copper deficiency. In general, all organs are affected but the brain is most sensitive to copper deficiency, and thus more severely affected. ATP7A facilitates the ATP fueled efflux in cells and the uptake of copper in the small intestine, and supplies the excreted copper-dependent enzymes with copper. This ATPase is localized in the trans-Golgi network [54, 55]. The copper deficiency, caused by failing copper-transport mechanisms leads to the classical form of MD and is fatal. Patients often die in the first years of life. However, there are milder phenotypes such as Occipital Horn Syndrome (OHS, MIM 304150 or X-linked cutis laxa), manifesting with cutis laxa, hernias, diverticula of the gastrointestinal tract and bladder, tortuosity of the arteries, hyperlaxity of the joints and skeletal anomalies. Compared to classical MD, the neurological symptoms are less severe and overall survival is longer. The term OHS is derived from the calcification of the attachments of the trapezius and sternocleidomastoid muscles to the occipital bone, forming occipital exostoses or occipital horns. These horns are visible on X-ray and make diagnostic differentiation from other MD phenotypes possible [5].

Since the initial description by Menkes, referred to as classical MD, many other symptoms have been associated with MD and milder forms have been described. Over 350 mutations in the *ATP7A* gene (not all published) leading to MD and OHS have been described to date [56]. MD and OHS form a spectrum in which clinical phenotypes are heterogeneous and genotype-phenotype relation is poor.

Diagnosis is based on clinical features, X-ray of the skull, decreased or low serum copper and ceruloplasmin levels. Genetic testing, by sequencing of the *ATP7A* gene is, however, the diagnostic gold standard [56]. So far, treatment options are limited, however, copper administration soon after birth in patients with residual ATP7A function seems promising and leads to improvement in survival [4]. Intrauterine copper treatment has been unsuccessful so far [57]. Experiments in mice with a combination of gene therapy and copper administration are also very promising. The effect on the brain is more pronounced and overall survival rates improved [58].

11.4.2 COG7-CDG

Congenital Disorders of Glycosylation (CDG) form a heterogeneous group of inborn errors of metabolism caused by defective biosynthesis of glycans. Since approximately half of our proteins are glycosylated, all having different functions and localizations, these glycosylation disorders are often multisystemic. The two main types of protein glycosylation comprise the *N*-glycosylation and the *O*-glycosylation. *N*-glycosylation, starting in the cytoplasm and endoplasmatic reticulum (ER), consists of the assembly of oligosaccharides onto a dolichol carrier. This process is completed in the Golgi where the processing and trimming of these assem-

bled glycans takes place. *O*-glycosylation, only consisting of processing and trimming, is limited to the Golgi. Defects in the *N*-glycosylation pathway usually lead to abnormal plasma tranferrin isoelectric focusing (TIEF) profiles due to the fact that transferrin is an *N*-glycosylated protein. Mucin type *O*-glycosylation defects lead to abnormal isofocusing of plasma apolipoprotein C-III (apoC-III) [59]. The protein *N*-glycosylation disorders can be subdivided in type I and II disorders based on the transferrin isoelectric focusing (TIEF) pattern.

COG7-CDG (CDG type IIe, MIM 608779) is caused by a mutation in subunit 7 of the COG complex existing of eight subunits in total [31, 60–62]. The COG complex is localized in the Golgi where it coordinates retrograde vesicle transport [63]. *COG7* defects result in a combined *N*- and *O*-glycosylation defect that can be diagnosed with an abnormal TIEF and apoC-III IEF. Most of the COG7-CDG patients described so far had a fatal outcome due to a common homozygous mutation. These patients were of consanguineous descent and suffered from severe growth retardation, microcephaly, hypotonia, hepatosplenomegaly, cardiac anomalies, recurrent infections, feeding problems, hyperthermia, adducted thumbs, mental retardation, seizures and structural brain abnormalities. All of these patients had abnormal fat distribution and cutis laxa or wrinkled skin. Elevated serum transaminases and bilirubin indicate abnormal liver function [31, 60–62]. COG7-CDG was the first link between glycosylation and cutis laxa [61]. To date no histological or electron microscopic evaluation is available indicating that abnormal elastin structure is the cause of the cutis laxa in COG7-CDG patients. One hypothesis is that hypoglycosylation of extracellular matrix proteins could be causative.

11.4.3 ATP6V0A2-CDG

Autosomal recessive cutis laxa type IIA (ARCL2A, Debré type MIM 219200) and Wrinkly Skin Syndrome (WSS; MIM 278250) are characterized by excessive skin wrinkling or congenital cutis laxa, intrauterine growth retardation, failure to thrive, microcephaly, multiple dysmorphic features, and a large and late closing fontanel [64]. Patients also suffer from hypotonia, feeding problems, hyperlaxity of joints and show abnormal fat distribution [25]. Strabismus and myopia are the most common ocular features. ARCL2A patients often show congenital hip dislocations and decreased bone mineral density. Developmental delay, mostly as a consequence of muscle hypotonia and joint laxity, is usually present. Seizures, hearing loss and cobble-stone like brain dysgenesis are frequently seen neurological features [29, 30]. The cutis laxa, unlike the other symptoms, seems to improve over time.

The initial finding of a combined N- and O-linked glycosylation defect in children with COG7-CDG, a Golgi transport defect, indicated the importance of glycosylation for extracellular matrix proteins. Therefore in a cohort of unsolved, consanguineous ARCL2 patients CDG screening was performed. All affected patients showed a type 2 pattern on transferrin isoelectrofocusing (TIEF), abnormal isoelectrofocusing of apolipoprotein C-III (apoC-III) and abnormal mass spectrometry of glycans of total serum proteins [25, 26]. Genetic analysis showed different loss of function mutations in the vesicular H^+-ATPase subunit a2, *ATP6V0A2* [26]. Since then several different mutations have been found to cause WSS and ARCL2. Genotype-phenotype correlation is poor [22]. The mechanism leading to cutis laxa in ATP6V0A2-CDG patients has yet to be fully elucidated. One of the hypotheses is that the v-ATPase related proton pump defect leads to abnormal transport of extracellular matrix (ECM) proteins [26, 27]. Other possibilities include delayed secretion of elastin and excessive storage of immature compounds like tropoelastin in the Golgi [22], and abnormal function and transport of glycosyltransferases leading to a hypoglycosylated state of diverse extracellular matrix components [22, 26].

11.4.4 P5CS

Δ^1-Pyrroline-5-carboxylate synthase (P5CS, MIM 138250) deficiency was first described in two Algerian siblings with progressive

neurodegeneration, joint laxity, skin hyperlaxity and bilateral subcapsular cataracts. A homozygeous missense mutation in *ALDH18A1*, the gene encoding P5CS, was discovered to be causative [65, 66].

P5CS catalyses the reduction of glutamate to Δ^1-pyrroline-5-carboxylate (P5C), a precursor of proline and ornithine. Ornithine is an important intermediate in the urea cycle and proline has an important role in protein synthesis. Deficiency of P5CS can lead to decreased levels of proline and ornithine.

Due to alternative splicing two isoforms of P5CS are generated: the short isoform, mainly participating in arginine production in the gut, and the long isoform responsible for proline synthesis in various tissues [70].

The clinical phenotype in P5CS deficiency is characterized by cutis laxa, microcephaly, bilateral subcapsular cataract, severe mental retardation, joint laxity and hypotonia, structural brain abnormalities, progressive neurodegeneration, seizures, peripheral neuropathy and dystonic movements in hands and feet. Biochemically, P5CS deficiency leads to a very typical pattern of paradoxical hyperammonemia as well as hypoprolinaemia and deficiency of urea cycle intermediates (ornithine, citrulline and arginine) [67].

The exact pathomechanism in which P5CS deficiency leads to cutis laxa or any of the other clinical symptoms remains unclear. Protein synthesis could be impaired in tissues highly depended on the endogenous production of proline such as collagen, thereby leading to cutis laxa [68]. However, if this was the case, an Ehlers-Danlos-like phenotype should be expected, and not cutis laxa. The presence of a proline transporter in synaptic vesicles and axon terminals of glutametergic neurons suggest a role of proline in neurotransmission. This function of proline could explain the clinical finding of severe mental retardation in P5CS deficient patients [69]. Proline has also been suggested to play a role as an anti-oxidant [70]. Decreased proline synthesis could therefore lead to increased apoptosis and developmental defects. Low levels of ornithine impair the urea cycle, and thus lead to low serum levels of citrulline and arginine, and hyperammonemia. Following a meal, dietary arginine will temporarily restore the urea cycle, explaining the paradoxical character of the hyperammonemia.

Bicknell et al. described a family with four members affected by autosomal recessive cutis laxa due to a C-terminal P5CS missense mutation [71]. Detailed biochemical serum analysis revealed only mildly lower ornithine levels under fasting conditions, but no significantly altered proline concentrations. Furthermore, the rate of proline biosynthesis from glutamate was normal in patient fibroblasts. In contrast, the clinical presentation was highly similar to the previously reported cases casting doubt on the role of proline synthesis itself in the disease mechanism.

Only a few patients have been reported, and evidence-based treatment options are thus very limited in P5CS defect. Trials with ornithine treatment showed poor results [67].

11.4.5 PYCR1

Pyrroline-5-carboxylate reductase 1 (PYCR1) catalyses the reduction of Δ^1-pyrroline-5-carboxylate (P5C) to proline, the final step in the synthesis of proline from glutamate [72]. Like P5CS, it plays a role in the biosynthesis of proline. PYCR1 is most highly expressed in bone and skin and is localized in mitochondria [33].

Clinical features in PYCR1 deficient individuals (MIM 179035) include cutis laxa (most pronounced on the dorsum of hands and feet), dysmorphic features, osteopenia, distal arthrogryposis, joint laxity, hernias, mild mental retardation, athetoid movements and aplasia or hypoplasia of the corpus callosum [33]. Whereas, based on the function of PYCR1, decreased serum proline levels would be expected, normal levels were found in patients [34]. Also in urine, low to normal levels of proline were found.

Guernsey et al. [35] described a homozygous mutation in the *PYCR1* gene, localized on chromosome 17, in five members of two Canadian families. This missense mutation, R266Q, leads to a premature termination of the PYCR1 gene product leading to loss of function [35]. Eight additional mutations have been found in 35 members of 22

families. *PYCR1* mutations have been found in various cutis laxa syndrome phenotypes, including De Barsy syndrome, Wrinkly Skin syndrome and also in ARCL2 patients, showing a similar clinical presentation as those with *ATP6V0A2* defect [33, 37].

Upon oxidative stress PYCR1-deficient fibroblasts showed low mitochondrial membrane potential, a disruption of the mitochondrial network, and increased apoptosis rates [33]. Knockdown of *PYCR1* in Xenopus and zebrafish entailed epidermal hypoplasia and blistering, accompanied by a massive increase in apoptosis [34]. Addition of exogenous proline did not influence these effects observed in vitro and in vivo. Although P5CS/PYCR1 defects are located in the same pathway, patients with *PYCR1* mutations are less severely affected and have a better prognosis due to the absence of neurological involvement. This can be explained by the ubiquitous presence of a highly identical paralogue, PYCR2. P5CS is encoded only by a single gene [33]. It remains unclear whether the proline synthesis is the major contributing factor to the pathomechanism in this disorder, comparable to P5CS. So far no treatment is available.

11.4.6 RIN2

Recently, a new elastic tissue disorder characterized by macrocephaly, alopecia, cutis laxa and scoliosis was discovered in three patients from two related consanguineous Israeli-Arab families [42]. The syndrome was termed MACS (MIM 613075). Other features included down-slanting palpebral fissures, puffy eyelids, sagging cheeks, everted lower lip, micrognathia and abnormal skull morphology, gingival hypertrophy, irregular dentition, sparse scalp hair and severe joint hyperlaxity. Psychomotor development was normal in all patients. Genetic analysis revealed a single base pair deletion in the *RIN2* gene on chromosome 20. Consequently, the same phenotype was described in three siblings from a consanguineous Algerian family [43]. A deletion in the *RIN2* gene was found to be causative in these patients.

The gene product of *RIN2*, the Ras and Rab interactor 2 protein, has been proven to interact with the Rab5 protein, which is required for endosomal trafficking [73]. Although endosomal trafficking itself was not impaired in RIN2-deficient patients, it has been suggested that RIN2-deficiency (MIM 610222) causes impairment of other trafficking pathways, e.g., ER-to-Golgi or Golgi-to-plasma membrane, thereby causing the phenotype [43]. Fibroblasts of the patients reported so far showed abnormal morphology of the Golgi apparatus with swollen cisternae and vacuole accumulation [33]. Biochemical investigations in one of the patients showed normal plasma *N*-glycans and variable alterations of the mucin type *O*-glycan Apolipoprotein C-III_1, similar to the profile seen in patients with abnormal Golgi-related *O*-glycosylation [44, 59, 74]. RIN proteins have also been described as regulators of the Ras-pathway [75]. This role in the Ras–pathway could explain the weak overlap between MACS syndrome and Costello syndrome (sparse hair, prominent lips, cutis laxa) (see below).

11.4.7 Gerodermia Osteodysplastica

Gerodermia osteodysplastica (MIM 231070), first described in 1950 by Bamatter et al, is a clinical syndrome characterized by lax, wrinkled skin, joint hyperlaxity, progeroid facial appearance, severe osteoporosis, malar and mandibular hypoplasia and growth retardation [76]. Due to the typical facial features the disorder was also termed ‘Walt Disney dwarfism’ [76]. Severe spontaneous fractures occur due to osteopenia and osteoporosis, and bisphosphonate therapy can have a positive effect in some patients [41]. In 2008 Hennies et al performed genetic analysis of affected individuals from 13 families. The study revealed nine pathogenic mutations in the *GORAB* (*SCYL1BP1*) gene [77]. The gene product of GORAB interacts with Rab6, a protein involved in intracellular trafficking [78]. Rab6 has been demonstrated to interact with the Conserved Oligomeric Golgi complex (COG) [79], surprisingly linking GO

with CDG. However, glycosylation disorders have not been observed in GORAB deficiency (MIM 607983) [77].

11.4.8 Metabolic Pathway Defects Described in Association with Wrinkled Skin or Cutis Laxa

The clinical presentation of transaldolase deficient patients is characterized by aortic coarctation, bleeding tendency, hepatosplenomegaly, telangiectases of the skin and enlarged clitoris. The genetic cause is a mutation in the *TALDO1* gene (MIM 602063) [80]. Biochemical investigations showed thrombocytopenia and elevated concentrations of ribitol, D-arabitol and erythritol in urine and plasma [80]. Mitochondrial involvement and the use of erythronic acid as a novel hallmark was recently described [81]. One of the patients with this rare condition also showed severe cutis laxa phenotype [82]. Until recently there was no evidence, whether the association of *TALDO1* defect and cutis laxa was a coincidence, and possibly a consequence of a second, associated genetic condition due to consanguineous decent of the patients. However multiple patients with *TALDO1* mutations have cutis laxa (2011, based on personal communications, Gajja Salomons). The exact mechanism of a pathophysiological link between these two disorders still needs to be elucidated.

Multiple heterozygous germ line mutations were found in genes encoding for enzymes and regulators of the RAS-MAPK pathway, thus causing Noonan syndrome (MIM 163950) and the related disorders Cardio-Facio-Cutaneous (MIM 115150), LEOPARD (MIM 151100 and 611554) and Costello syndrome (MIM 218040) [83]. Mutations in *PTPN11, RAF, NF, HRAS, BRAF, MEK1/2, KRAS*, *SOS, SHOC2, NRAS* and *RAF1,* all regulating the Ras-MAPK cascade, have all been identified in patients with these syndromes [84]. Clinically they are characterized by distinctive facial appearance, heart defects, ectodermal abnormalities, variable cognitive defects, psychomotor developmental delay and susceptibility to certain malignancies. Several of the patients with Noonan, Cardio-Facio-Cutaneous or Costello syndromes have been reported to have wrinkled skin and cutis laxa, especially in the inguinal, axillary regions, and affecting the hands and feet [46].

11.5 Histological Abnormailities in Cutis Laxa Syndromes

11.5.1 Autosomal Dominant Cutis Laxa

In autosomal dominant (AD) *ELN* mutations, a reduced overall amount or complete absence of elastic material and fragmented elastic fibers were found with light and electron microscopy [8, 47, 85]. Subsequently an increasing electron density from the inner to outer regions of the elastic material was found [8, 47]. The shape of elastin fibers in the reticular (lower) dermis was highly abnormal, and some fibers even showed a ‘moth-eaten’ appearance [8]. The amorphous component of elastic fibers showed extensive fragmentation and branching [8, 47]. Fragmented fibers were also noted [85]. An excess of microfibrils compared to amorphous elastin was found by Callewaert et al. [47] and Graul-Neumann et al. [85], but not discovered by Tassabehji et al. [8]. The other way around, a loose matrix was only reported by Tassabehji et al. [8]. Patients with an autosomal dominant *ELN* mutation and only mild histological findings were also reported [47, 85]. To our knowledge, there is only one article reporting histology in patients with AD *FBLN5* mutations. With light microscopy, an increase in *FBLN5* immunoreactivity can be recognized by short plump fibers, more pronounced in the lower dermis. Elastic fibers were fragmented, short, generally absent in the papillary (upper) dermis and reduced in the lower dermis. With electron microscopy, it was also seen that the elastic fibers were small in size and disorderly shaped. Elastotubules were prominent and appeared to be detached from the elastin. A finding unique for AD *FBLN5* mutations is the presence of abnormal collagen fibers consisting of small fibrils, some in twisted, rope like arrange-

ment. However, patients with milder histological findings or even normal amounts and sized elastic fibers were also reported [49].

11.5.2 X-Linked Cutis Laxa

Only one article reports histology results in patients with an X-linked *ATP7A* mutation, Occipital Horn Syndrome or Menkes Disease. Rare fragmented and small elastic fibers and an excess of microfibrils were reported. Unique for this type of cutis laxa are dense bundles of collagen fibers and large fibroblasts [86].

11.5.3 Autosomal Recessive Cutis Laxa

Comparable to the clinical features, the histological findings in patients with ARCL1 are also very severe. Paucity, fragmentation, decreased density and severe underdevelopment of elastic fibers have been reported in *FBLN4* patients [20, 21]. In addition, smaller-than-normal collagen bundles and increased vascularization in the upper dermis have also been reported [20]. In ARCL1 patients with a *FBLN5* mutation, severely underdeveloped elastin fibers with a marked gross granular appearance or even a complete absence of visible elastic fibers have been found [16, 17], In contrast to patients with AD *ELN* mutations, microfibrils were scarce in these patients. Overall the extracellular matrix displayed a low density and loose organization and disorganized collagen fibers have been described. The lamina densa in the skin of patients with an autosomal recessive *FBLN5* mutation was thinner compared to the skin of controls [17]. So far no histological findings in ARCL caused by *ELN* mutations have been reported [87].

In ARCL1 patients with *LTBP4* mutations, elastic fibers in the upper dermis were absent. Dermal elastic fibers were fragmented, weakly stained and showed less-defined edges. Unique for this type of cutis laxa is the considerable amount of elastin located external to the bundles in large, globular deposits [88].

In ARCL2 patients with an *ATP6V0A2* mutation, only general defects of the extracellular matrix, such as decreased amount of elastin fibers and fragmented elastic fibers have been reported [25, 29]. More distinctive features shown by EM are abnormal swelling and fragmentation of the Golgi apparatus, and the presence of excessive lysosomes, autophagosomes and multivesicular bodies in the fibroblasts [22]. In patients with a *PYCR1* mutation, sparse, small, abnormally thin and fragmented elastic fibers have been described with jagged or cribriform contours [34, 89, 90]. Curiously, the scarcity of elastic fibers was most pronounced in the reticular dermis of patients [89]. A reduction in both the fibrillar and amorphous components of elastic fibers was reported by Reversade et al. [34] and Kretz et al. [90] described excessive microfibril contours. Characteristic for *PYCR1* mutations is the altered morphology of mitochondria and their cristae [34, 89, 90]. A higher number of inflammatory cells were seen around vessels in the papillary dermis and excessive lipid droplets in the cytoplasm compared to control fibroblasts. Collagen bundles were scattered, less compact, had irregular contours, variable sized diameters and also showed variation in single fibril calibers [89, 90]. Both *P5CS*- and *PYCR1*-deficiency patients had abnormally large lysosomes in dermal fibroblasts, but in contrast to *PYCR1* patients, *P5CS* patients had normal mitochondria morphology [39]. Patients with cutis laxa due to a *P5CS* mutation also had a rarefaction and a decrease in the size of the elastic fibers, but in contrast to elastic fibers of *PYCR1* patients, elastic fibers of *P5CS* patients showed no jagged contours [39, 40]. Similar to cutis laxa patients with an AD *ELN* mutation, elastic fibers of *P5CS* patients appeared to be moth-eaten. Both Martinelli et al. and Skidmore et al. found some collagen abnormalities with light microscopy: collagen bundles were of variable diameters and appeared less well defined and lacked the characteristic wavy morphology. However, with electron microscopy, Martinelli et al. found irregular contours and variable sizes in diameters of collagen fibrils, whereas Skidmore et al. only found a decreased number of collagen fibers with an otherwise

unaffected morphology [39, 40]. The dermis of *P5CS* patients was thinner compared to that of healthy controls [39].

In gerodermia/geroderma osteodysplastica patients, with ARCL2 due to *GORAB* mutations, a reduced amount or absence of elastic fibers in the papillary dermis have been described. Fragmentation and clumping of elastic fibers in the reticular dermis were noted [91, 92]. With electron microscopy, Nanda et al found that elastic fibers were widely spaced with fenestration and clumping of the microfibrillar component [91]. Del Carmen Boente et al., however, reported that the majority of the elastic fibers showed a normal morphology with a normal fibrilar/amorphous material relationship. Only in some areas, elastic fibers were fragmented [92]. In cutis laxa patients with *RIN2* mutations, elastic fibers were sparse in the papillary dermis, but were normally distributed in the reticular (lower) dermis [92]. There was also a diminished microfibrillar component at the periphery of elastic fibers [42]. Syx et al. reported that numerous collagen bundles contained variable diameter fibrils and irregular interfibrillar spaces enriched in granulo-filamentous deposits [43], but Basel-Vanagaite et al reported a normal structure and distribution of collagen throughout the skin [42]. Syx et al. also reported that fibroblasts exhibited a dilated endoplasmic reticulum (ER) and an abnormal Golgi apparatus with rarefied and dilated cisternae [43]. Minimal focal interstitial mucin deposition was detected with Alcian blue staining and osmiophilic structures were abundant in the amorphous matrix in some elastic fibers in the reticular dermis [42, 43]. This might indicate an inflammatory process.

MAP-K pathway deficiencies and *TALDO1* mutations have only recently be discovered as causes of wrinkled skin and cutis laxa, therefore histological characteristics of these patients remain unreported.

11.5.4 Differential Diagnosis

Increasing electron density from the inner to the outer regions of elastin fibers is found specifically in patients with autosomal dominant *ELN* mutations [8, 47]. However, diminished microfibrillar component at the periphery of elastic fibers can be found in patients with autosomal recessive *RIN2* mutations [42]. Extensive fragmentation and branching of the amorphous component of elastic fibers can also be found in patients with *ELN* mutations [8, 47]. Elastotubules that appear to be detached from the elastin have been reported in patients with ADCL caused by *FBLN5* mutations [49]. Elastin located external to the bundles in globular deposits is specific for AD *ELN* and AR *LTBP4* mutations [47, 88]. Disorderly shaped elastic fibers can be found in patients with both types of ADCL [8, 44]. A moth-eaten appearance of elastin can be found both in patients with autosomal dominant *ELN* mutations [8] and in those with autosomal recessive *P5CS* mutations [40]. Unique for X-linked cutis laxa are the dense bundles of collagen fibers and large fibroblasts [96]. Elastic fibers are almost absent or severely underdeveloped in patients with the severe type 1 autosomal recessive cutis laxa, caused by *FBLN4* and *FBLN5* mutations [16, 17, 20]. The extracellular matrix of AR *FBLN5* patients has been reported to be very low-dense as a whole [17]. Abnormally thin elastic fibers with jagged or cribriform contours seem to be specific for *PYCR1* mutations [34, 89, 90]. Smaller-than-normal collagen bundles have been reported in *FBLN4* patients [20].

A twisted, rope-like arrangement of collagen is characteristic for AD *FBLN5* mutations [49]. Variably sized diameters of collagen fibrils have been found in patients with *PYCR1*, *P5CS* and *RIN2* mutations [39, 43, 90]. Irregular contours of collagen fibrils have been found in *PYCR1* and *P5CS* patients [39, 90]. Collagen bundles were also scattered and with less compact in *PYCR1* patients [89]. In *RIN2* patients, numerous collagen bundles contained irregular interfibrillar spaces enriched in granulo-filamentous deposits [43].

Features that do not relate to the extracellular matrix are abnormal swelling and fragmentation of the Golgi apparatus and redundant lysosomes, autophagosomes and multivesicular bodies in the fibroblasts of patients with *ATP6V0A2* mutations

Table 11.1 Differential diagnosis of gene defects in cutis laxa, based on histological findings

Inheritance pattern	Mutation	Distinctive elastic fiber features	Collagen abnormalities?	Other features
AD	ELN	An increasing electron density from the inner to outer regions of the elastic material	–	Also patients with mild histological features
		Extensive fragmentation and branching of amorphous component		
		Moth-eaten appearance		
AD	FBLN5	Short plump fibers, more pronounced in the lower dermis	++	Also patients with mild histological features
X-linked	ATP7A	–	–	Dense bundles of collagen fibers and large fibroblasts
AR	FBLN4	Severe underdevelopment	+	–
	FBLN5	Severe underdevelopment or even absence	+	Microfibrils are scarce
				The extracellular matrix displays a low density and loose organization
				Thinner lamina densa
	LTBP4	Less-defined edges	–	
		Considerable amount of elastin located external to the bundles		
	ATP6V0A2	–	–	Abnormal swelling and fragmentation of the Golgi apparatus
				Many lysosomes, autophagosomes and multivesicular bodies in the fibroblasts
	PYCR1	Abnormally thin with jagged or cribriform contours	+	Altered morphology of mitochondria and their cristae
				Abnormally large lysosomes
				Inflammatory
	P5CS	Moth-eaten appearance	++	Abnormally large lysosomes
				Thinner dermis
	GORAB	Clumping of (the microfibrillar component of) elastic fibers	–	–
		Majority with normal morphology		
	RIN2	Also normal fibers	+/–	Dilated endoplasmic reticulum (ER)
		Diminished microfibrillar component at the periphery		An abnormal Golgi apparatus with rarefied and dilated cisternae
				Possibly inflammatory

[22]. Altered morphology of mitochondria and their cristae has been seen in *PYCR1* patients [34, 89, 90]. Both *P5CS*- and *PYCR1*-deficiency patients had abnormally large lysosomes in dermal fibroblasts [39]. Finally, a dilated endoplasmic reticulum (ER) and abnormal Golgi apparatus with rarefied and dilated cisternae have been reported in *RIN2* patients [43]. For an overview of histological findings see Table 11.1.

11.6 Multiple Malformation Syndromes Associated with Cutis Laxa

There are several autosomal recessive multiple malformation syndromes, and a few autosomal dominant conditions, associated with wrinkled skin or cutis laxa. The patients affected with these syndromes do not always present with

skin involvement. These syndromes have usually other distinctive features suggesting the correct diagnosis and the presence of wrinkled skin is not among the major diagnostic criteria.

One of these syndromes is Cantu syndrome (MIM 114620), a skeletal dysplasia with short stature, ovoid vertebral bodies and metaphyseal flaring of the long bones with metacarpal and phalangeal anomalies. Patients have cardiac rhythm anomalies and cardiomyopathy, hypertrichosis and a recognizable face, in association with cutis laxa in some of the patients. Patients have been recently described with mutations in the *ABCC9* gene, coding for a membrane ATPase, similar to the pathomechanism and gene defect underlying pseudoxanthoma elasticum (MIM 264900), caused by mutaions in the *ABCC6* gene [93]. Pseudoxanthoma elasticum is an autosomal recessive condition with a progressive clinical picture of xanthomatosis (yellow plaque formation) in the nuchal and axial region, in the flexor surface of the elbow and in the popliteum associated with local elastolysis. Patients develop a severe cardiovascular disease with angioid streaks [94].

Kabuki syndrome (MIM 147920) is a syndrome of severe growth deficiency, developmental delay and characteristic eyes with partial eversion of the lower eyelid and prominent dark whimpers, similar to the makeup used in the past by Kabuki theater actors. Patients commonly have cardiac and renal anomalies, bifid uvula or cleft palate and epilepsy. Cutis laxa might be present in the inguinal and axial region or around the eyes or dorsum of the hands. Recently a novel genetic pathway was identified in a significant number of patients with Kabuki syndrome (*MILL2*), showing genetic heterogeneity [95].

Keutel syndrome is an autosomal recessive condition (MIM 245150) associated with hearing loss and progressive cartilaginous ossification of the pinnae, larynx and sometimes also calcifications in brain, recurrent episodes of otitis media, epilepsy with variable mental retardation, cardiac anomalies and cutis laxa in the minority of patients [96]. Keutel syndrome is caused by gene defects in the *MGP* gene.

In some other multiple malformation syndromes associated with wrinkled skin or cutis laxa the genetic background has not yet been unraveled. GAPO syndrome (MIM 230740) is similar to MACS syndrome, sharing alopecia and oligodontia, facial dysmorphia and growth delay, and in both disorders there is a facial predominance of cutis laxa, however in GAPO syndrome patients show optic atrophy and glaucoma, discriminating the two disorders [97].

SCARF syndrome (MIM 312830) is such a disease with a so far unknown genetic origin. The diagnosis is based on the presence of symptoms underlying the acronym; skeletal anomalies, craniosyntostosis, ambigous genitalia, retardation and facial anomalies. Most patients with skin involvement have wrinkled skin but not severe cutis laxa [98]. Barber-Say syndrome (MIM 209885) is a similar entity with ambigous genitalia and hypertrichosis and facial cutis laxa with eye anomalies. Severe eye anomalies dominate the clinical picture of Ablepharon macrostomia syndrome (MIM 200110) and this syndrome is also associated with ambigous genitalia. The underlying gene defect has not been discovered in this syndrome either, and one might propose that these three conditions with ambiguous genitalia, facial anomalies, eye symptoms and facial cutis laxa could be different manifestations of the same disease with variable expression and severity [99].

Additional to the presence of wrinkled, loose skin there are a few other syndromes with progeria, sharing some of the other, progerioid skin features with autosomal recessive cutis laxa type II. One of these disorders is the dominantly inherited Hutchinson Gilford progeria (MIM 176670), a progressive genetic disorder of tissue specific glycosylation, caused by mutations in the *LMNA* gene. Patients develop generalized progeria, pergament-like, thin skin with almost no subcutaneous fat under the skin, prominent veins, alopecia, carries and early skin wrinkling. Parallel with the abnormal skin aging the whole body undergoes premature aging, in association with hypercholesterinemia, artherosclerosis, cardiovascular disease and joint stiffness. Patients die also at a young age [100]. Premature aging is present in

amyloidosis, including the inherited type (MIM 105120) associated with corneal abnormalities and polineuropathy, caused by *GSN* mutations. However, cutis laxa in this case is secondary, similar to Hutchinson Gilford progeria, and progressive. Progerioid, atrophic skin and prominent subcutaneous veins has been described in Lenz-Majewski hyperostotic dwarfism (MIM 151050) and Wiedemann Rautenstrauch syndrome (MIM 264090) as well, in the first in association with a severe skeletal dysplasia and in the latter in association with peculiar face and profound developmental delay in patients [101].

11.7 Differential Diagnosis in Cutis Laxa Syndromes

The differential diagnosis of cutis laxa includes wrinkled skin and a few other excesses of skin disorders unrelated to cutis laxa syndromes. Excessive skin can be observed in several collagen disorders, especially in Ehlers Danlos syndrome type I and VI (MIM 130050, MIM 225400) (X). Furthermore, severe wrinkling could appear after severe weight loss (especially, in babies with dysmaturity at birth and in case of rapid weight loss due to failure to thrive in the first months of life), in lipodystrophy syndromes and in diverse progeria syndromes. In all of these cases a thorough history (hyperlaxity of the skin with bruises and scarring, scoliosis, excessive weight loss, severe failure to thrive and progressive skin wrinkling in association with other signs of getting prematurely old) would help in the differential diagnosis.

True cutis laxa has a decreased elasticity of the skin, presenting early in the course of the disease, and is not associated with bruising and scarring, as in Ehlers Danlos syndrome patients. In case of uncertainty one should perform skin biopsy to confirm abnormal elastin structure in the skin. We should also note that in some cases of severe wrinkled skin differentiating collagen disorders from cutis laxa is not so straightforward, since collagen fibers can be abnormal in several types of true cutis laxa as well, additional to the typical elastin abnormalities [90].

The number of genetically solved cutis laxa syndromes has steadily risen in recent years (see Table 11.2). Due to overlapping symptoms, it is not easy to suggest a diagnostic strategy. The question remains, how can we find which gene is mutated in the most efficient way? As mentioned clinical features might be overlapping; however, there are a few discriminative symptoms for each syndrome. Combination of clinical information, biochemical analyses and cranial imaging in cutis laxa patients will facilitate guided mutational analyses and a quick correct diagnosis (see diagnostic chart Fig. 11.5).

Careful history taking with emphasis on family history and disease onset should distinguish acquired cutis laxa from an inherited syndrome. In addition, the information gained from family history and presence or absence of severe systemic involvement will help to differentiate between the inheritance patterns. XRCL and ARCL type 1 patients often present with severe systemic involvement [102].

Analyses of copper and ceruloplasmin levels and a skull X-ray should be performed when suspecting XRCL. Low copper and ceruloplasmin levels and cranial exostoses are diagnostic of XRCL, due to *ATP7A* mutations ([4]; Kaler et al. 1994). Physical examination in combination with detailed neurological evaluation should guide mutational analysis in ARCL2 [1, 30]. Patients with ARCL2A (*ATP6V0A2)* or ARCL2B (*PYCR1)* have a large, late closing fontanel, short noses and long philtrums. ARCL2A patients furthermore have down-slanting palpebral fissures and generalized cutis laxa (although improving over time) [1, 25, 26, 30]. In contrast, PYCR1 patients have a more pergament-like skin, with wrinkling most pronounced on hands and feet [34, 38, 90, 103]. GORAB patients often have a history of multiple fractures and skeletal dysplasia (including post fractural abnormalities) is visible upon radiographic evaluation. Most GORAB patients have normal intellect [77]. MACS syndrome patients (*RIN2)* have a characteristic coarse appearance of the face with full lips and a sagging chin. They suffer from severe scoliosis [42, 43]. Several forms of the cutis laxa syndromes are presenting with muscle hypotonia

Table 11.2 Differential diagnostic features of heritable forms of cutis laxa and related disorders

Disorder	Abbreviation	Skin abnormalities	Metabolic changes	Discriminative features	Mutation
Autosomal dominant cutis laxa	ADCL	Generalized, not always present at birth	–	Pulmonary and cardiovascular manifestations absent, can be inherited, milder or later onset	*ELN*
Autosomal dominant cutis laxa (macular degeneration with skin wrinkling)	ADCL	Generalized, mild, not always present at birth	–	Macular degeneration	*FBLN5*
X-linked recessive cutis laxa Occipital horn syndrome	XLCL OHS	Generalized, severe, inguinal sagging, present at birth	Abnormal copper metabolism	Occipital exostoses, cardiovascular involvement, hypotonia, pili torti	*ATP7A*
Autosomal recessive cutis laxa type 1A	ARCL1A	Generalized, severe skin sagging, present at birth	–	Arterial tortuosity, lethal pulmonary hypertension, bone fragility	*FBLN4*
Autosomal recessive cutis laxa type 1B	ARCL1B	Generalized, severe skin sagging, present at birth	–	Supravalvular aortic stenosis, lethal developmental emphysema	*FBLN5*
Autosomal recessive cutis laxa type 1C	ARCL1C	Generalized skin sagging, present at birth	–	Severe gastrointestinal and urinary malformations, lethal developmental emphysema mild cardiovascular involvement	*LTBP4*
Autosomal recessive cutis laxa type 2A	ARCL2A	Generalized, severe skin sagging, present at birth, improving with time	Abnormal N-linked and O-linked glycosylation	Growth and developmental delay, abnormal glycosylation of serum proteins	*ATP6V0A2*
Autosomal recessive cutis laxa type 2B	ARCL2B	Generalized, progerioid, pergament like skin, less sagging, present at birth	High ammonia, abnormal amino acid pattern	Growth and developmental delay, triangular face, prominent veins, normal glycosylation	*PYCR1, ALDH18A1*
De Barsy syndrome	DBS/ARCL3	Generalized, progerioid, pergament like skin, less sagging, present at birth	High ammonia, abnormal amino acid pattern	Corneal clouding, athetoid movements	*ATP6V0A2, PYCR1, ALDH18A1*
Gerodermia osteodysplastica	GO	Generalized, severe skin sagging, present at birth	Abnormal N-linked and O-linked glycosylation	Bone fragility, short stature	*GORAB*
Macrocephaly Alopetia Cutis Laxa Scoliosis syndrome	MACS	Sagging chin, wrinkled neck skin present at birth	Abnormal O-linked glycosylation	Macrocephaly, alopecia, scoliosis Coarsening facial features	*RIN2*
Arterial Tortuosity syndrome	ATS	Mild, progerioid skin, less sagging, present at birth	–	Triangular face, arterial tortuosity, normal lungs	*SLC2A10*

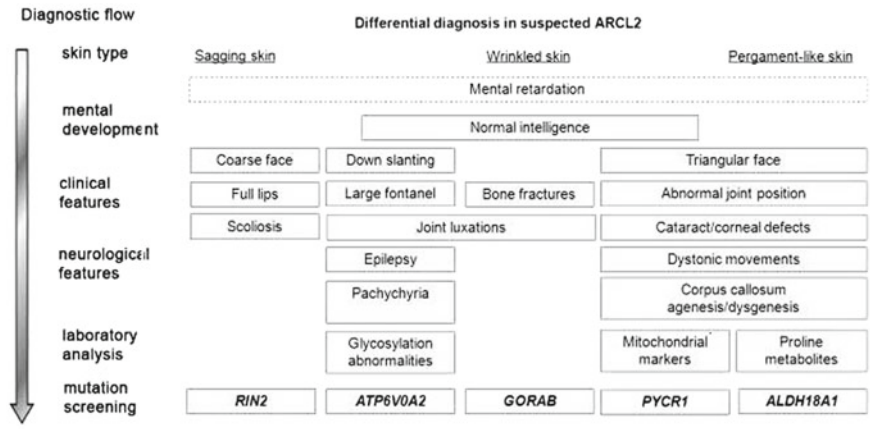

Fig. 11.5 Diagnostic flow-chart in suspected ARCL2

(*ATP6V0A2, ATP7A* and *PYCR1* defects), spasticity (*PYCR1* and *ALDH18A1* defects), dystonic, athetotic movements (*ALDH18A1* and *PYCR1* defect) or epilepsy (*ALDH18A1, ATP6V0A2* and *ATP7A* defects) [77, 90]. Developmental delay is frequent in patients with *ATP6V0A2* and *PYCR1* mutations [25, 34]. To date no neurological involvement has been described in GO patients carrying *GORAB* mutations and most of the patients showed normal mental development as well [77]. Cobblestone brain dysgenesis is often seen on cranial imaging in patients with ATP6V0A2-CDG [22, 29]. MRI abnormalities of the corpus callosum are common in patients with ARCL2B [34].

In addition to these clinical markers, metabolic alterations might give important clues, since they are surprisingly common [33]. Abnormal glycosylation screening is found in patients with COG7 – en ATP6V0A2-CDG [25, 30, 61]. Alanin and lactate levels, markers for mitochondrial dysfunction, have been found in a subgroup of patients with mutations in *PYCR1* [34]. In patients with a deficiency of *ALDH18A1* decreased levels of proline and ornithine have been described in addition to a paradoxical hyperammoniemia [71].

In summary, if specific clinical features are present, for example, scoliosis with alopecia (*RIN2*) or frequent bone fractures (*GORAB*), seizures associated with improving cutis laxa pattern with aging (*ATP6V0A2*) or dystonia in association with cutis laxa on hand and feet (*PYCR1*) direct mutation analysis can be performed. In other cases glycosylation studies, measurement of blood lactate, ammonia and serum amino acids, should be performed in addition to performing a MRI.

Although in recent years many new genes have been found to cause cutis laxa, there is a large group of patients in which the underlying genetic defect has yet to be discovered.

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Collagen Type VI Myopathies

12

Kate M.D. Bushby, James Collins, and Debbie Hicks

Abstract

Mutations in each of the three collagen VI genes COL6A1, COL6A2 and COL6A3 cause two main types of muscle disorders: Ullrich congenital muscular dystrophy, a severe phenotype, and a mild to moderate phenotype Bethlem myopathy. Recently, two additional phenotypes, including a limb-girdle muscular dystrophy phenotype and an autosomal recessive myosclerosis reported in one family with mutations in COL6A2 have been reported. Collagen VI is an important component of the extracellular matrix which forms a microfibrillar network that is found in close association with the cell and surrounding basement membrane. Collagen VI is also found in the interstitial space of many tissues including muscle, tendon, skin, cartilage, and intervertebral discs. Thus, collagen VI mutations result in disorders with combined muscle and connective tissue involvement, including weakness, joint laxity and contractures, and abnormal skin findings.

In this review we highlight the four recognized clinical phenotypes of collagen VI related – myopathies; Ullrich congenital muscular dystrophy (UCMD), Bethlem myopathy (BM), autosomal dominant limb-girdle muscular dystrophy phenotype and autosomal recessive myosclerosis. We discuss the diagnostic criteria of these disorders, the molecular pathogenesis, genetics, treatment, and related disorders.

Keywords

Mutations in collagen VI genes *COL6A1* • *COL6A2* and *COL6A3* • Ullrich congenital muscular dystrophy • Bethlem myopathy

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Abbreviations

AD	Autosomal Dominant
BM	Bethlem Myopathy
CCD	Central Core
CGH	Comparative Genomic Hybridization
CMD	Congenital Muscular Dystrophy
CsA	Cyclosporine A
DGC	Dystrophin-Glycoprotein Complex
ECM	Extracellular Matrix
EDMD	Emery-Dreifuss Muscular Dystrophy
EDS	Ehlers-Danlos Syndrome
EMG	Electromyography
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
LGMD	Limb Girdle Muscular Dystrophy
MDC1A	Merosin-Deficient Congenital Muscular Dystrophy Type 1A
MDC1C	Congenital Muscular Dystrophy Type 1C
MDC1D	Congenital Muscular Dystrophy Type 1D
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
PTP	Permeability Transition Pore
siRNA	Short Interfering Ribonucleic Acid
SR	Sarcoplasmic Reticulum
TGF-β	Transforming Growth Factor beta
UCMD	Ullrich Congenital Muscular Dystrophy
vWFA	von Willebrand Factor Type A

12.1 Collagen VI Related: Myopathies

Mutations in each of the three collagen VI genes COL6A1, COL6A2 and COL6A3 cause two main types of muscle disorders: Ullrich congenital muscular dystrophy (UCMD), a severe phenotype, and a mild to moderate phenotype Bethlem myopathy (BM). Recently, two additional phenotypes, including a limb-girdle muscular dystrophy phenotype and an autosomal recessive myosclerosis reported in one family with mutations in COL6A2 have been reported. Collagen VI is an important component of the extracellular matrix which forms a microfibrillar network that is found in close association with the cell and surrounding basement membrane. Collagen VI is also found in the interstitial space of many tissues including muscle, tendon, skin, cartilage, and intervertebral discs. Thus, collagen VI mutations result in disorders with combined muscle and connective tissue involvement, including weakness, joint laxity and contractures, and abnormal skin findings.

In this review we highlight the four recognized clinical phenotypes of collagen VI related – myopathies; Ullrich congenital muscular dystrophy, Bethlem myopathy, autosomal dominant limb-girdle muscular dystrophy phenotype and autosomal recessive myosclerosis. We discuss the diagnostic criteria of these disorders, the molecular pathogenesis, genetics, treatment, and related disorders.

12.1.1 Clinical Presentation

12.1.1.1 Ullrich Congenital Muscular Dystrophy

UCMD usually presents congenitally but there may be milder intermediate phenotypes approaching what would classically be described as Bethlem myopathy. While the two disorders are usually clearly clinically distinct, it is important to recognize that this is a continuum of clinical severity with two predominant phenotypes. Major clinical features of UCMD are shown in Table 12.1. Classically UCMD patients present in the newborn period with significant weakness and hypotonia. They may also present with congenital hip dislocation and contractures including torticollis, kyphoscoliosis, and proximal joints (Fig. 12.1h). Concomitantly there is marked laxity of the distal joints, including fingers, wrists, and ankles [51, 57] (Fig. 12.1i–k). Contractures and kyphosis may be initially transient but later recur with spinal rigidity, progression of contractures, and distal joints giving way to long finger flexion and Achilles tendon contractures. Progression of spinal rigidity and scoliosis is common and may require surgical correction. Posterior protruding calcaneus (Fig. 12.1g), rounded face, and drooping of lower eyelid are

Table 12.1 Ullrich congenital muscular dystrophy diagnostic criteria

Family history
Autosomal recessive, autosomal dominant inheritance, and de novo mutations
Neonatal features
Hypotonia, distal joint laxity, hip dislocation, contractures, kyphoscoliosis, torticollis
Age of onset
Neonatal to infancy
Motor skills and weakness
Delayed motor milestones
Do not achieve ambulation or loss of ambulation in first decade
Proximal to generalized muscle weakness, slowly progressive
Generalized muscle atrophy
Early respiratory failure
Connective tissue involvement
Distal laxity: fingers, wrists, ankle
Proximal joint contractures early on including spine, followed by distal joint contractures
Rounded face, lower eye lid lag, prominent ears
Prominent calcaneus
Skin features
Hypertrophic scar, keloid, extensor surface hyperkeratosis pilaris
Other features
Proportion of patients with failure to thrive
No central nervous involvement
Normal cardiac function
Laboratory criteria
Serum creatine kinase: normal to mildly elevated
Muscle pathology
Myopathic to dystrophic
Muscle and cultured fibroblast collagen VI staining
Abnormal
Muscle imaging
Peripheral increased signal with relative central sparing of the central part of the involved muscle. Rectus femoris demonstrating the "central shadow"
Collagen VI gene mutation: *COL6A1*, *COL6A2*, *COL6A3* genes

also common clinical features. There are abnormalities of the skin present that include soft velvety skin on the soles and palms, hyperkeratosis pilaris on the extensor limb surfaces and abnormal development of hypertrophic, keloid, scar formation (Fig. 12.1f). Delayed motor milestones are variable. In severe cases, independent ambulation is not achieved or loss of independent ambulation occurs in early childhood through the first decade. Intelligence and cardiac evaluations are normal. However, respiratory insufficiency occurs within the first and second decade, with diaphragmatic weakness sometimes out of proportion to skeletal muscle weakness, requiring noninvasive ventilatory support [73]. There is frequent failure to thrive necessitating nutritional supplementation [52].

12.1.1.2 Bethlem Myopathy

Bethlem myopathy is at the mild end of the clinical spectrum (Table 12.2). The age of onset of Bethlem myopathy is much more variable. Patients may present congenitally with decreased fetal movements, neonatal hypotonia, arthrogryposis, congenital hip dysplasia and torticollis. More frequently onset is in early childhood with

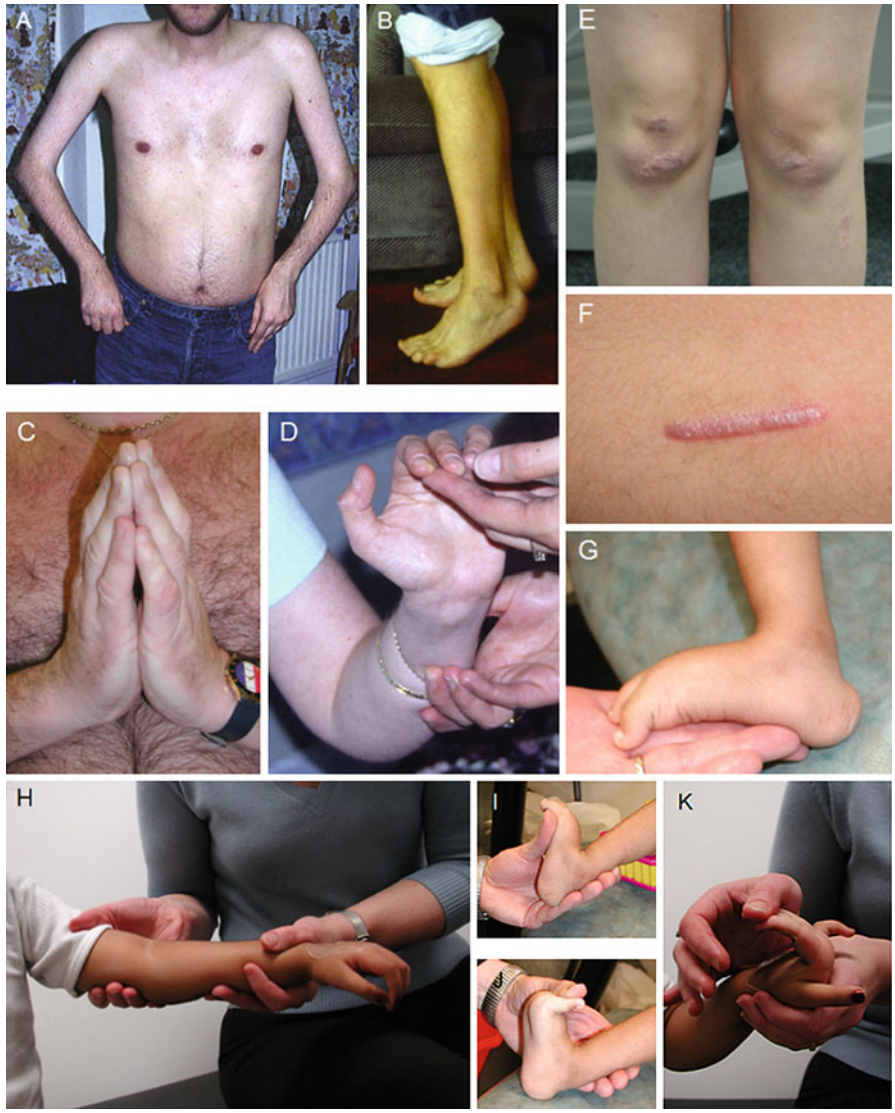

Fig. 12.1 Characteristic clinical features of BM (**a**–**e**) and UCMD (**f**–**k**). BM patients typically show flexion contractures of the fingers, wrists, elbows, and ankles (panels **a**–**d**) and keloid formation (panel **e**). In UCMD, proximal joint contractures (panel **h**) and striking hyperelasticity of the distal joints (panels **i**–**k**) are commonly seen, as are posteriorly protruding calcanei (**g**). As in BM, keloid scarring is also common (**f**) (From Lampe and Bushby [43]. http://jmg.bmj.com/content/42/9/673.full?sid=3fee0735-53e2-4e9e-b6a6-f8d32452e26c)

mild developmental delay, evidence of proximal weakness, difficulty with sports, and contracture development, often with toe walking evident. However onset in adulthood can occur, as late as the sixth decade [66]. The progression is slow, and after the fifth decade about half of the patients need ambulatory support [57]. The patients have mild to moderate weakness and atrophy of the muscles of the trunk and limbs, the proximal muscles being more involved than the distal muscles, and the extensors more than the flexors. Neck flexion weakness and mild facial weakness can occur. The hallmark of the disease is the presence of flexion contractures of the elbows, wrists, ankles and interphalangeal joints of the last four fingers [4, 50] (Fig. 12.1a–d). Joint contractures

Table 12.2 Bethlem myopathy diagnostic criteria

Family history
Autosomal dominant (predominant), autosomal recessive
Neonatal features
Hypotonia, distal joint laxity, hip dislocation, contractures, torticollis
Age of onset
Usually childhood to early adulthood
Motor skills and weakness
Mild to no developmental motor delay; all achieve ambulation
Proximal (most) to generalized muscle weakness, slowly progressive
Muscle atrophy predominantly shoulder girdle and lower legs
Connective tissue involvement
Distal laxity may be present especially at a younger age
Joint contractures develop predominantly Achilles tendon, long finger flexor, elbow and spine
Skin features
Hypertrophic scar, keloid, extensor surface hyperkeratosis pilaris
Other features
No central nervous involvement
Normal cardiac function
Respiratory function typically normal until 4th or 5th decade
Laboratory criteria
Serum creatine kinase: normal to mildly elevated
Muscle pathology
Myopathic to dystrophic
Muscle collagen VI staining
Usually normal
Cultured fibroblast collagen VI staining
Usually abnormal
Muscle imaging
Peripheral increased signal with relative central sparing of the involved muscle. Rectus femoris demonstrating the "central shadow" sign
Collagen VI gene mutation: *COL6A1*, *COL6A2*, *COL6A3* genes

of the jaw, shoulder, hip, knees, foot (pes cavus) and spine rigidity may also occur. Hyperlaxity of some joints in the early stages can be present but later evolve into contractures. Often skin abnormalities such as hypertrophic scar or follicular hyperkeratosis are evident (Fig. 12.1e). Usually the cardiac system is spared and respiratory involvement may not be evident until the fifth or sixth decades [26].

12.1.1.3 Autosomal Dominant Limb Girdle Muscular Dystrophy Phenotype

Collagen VI – related myopathy with an autosomal dominant limb girdle muscular dystrophy (AD LGMD) phenotype was first reported in three large families [64]. Patients demonstrated variable onset from infancy to adulthood. Proximal greater than distal skeletal muscle weakness was noted to varying degrees. However, minimal connective tissue involvement was seen. Joint contractures were either absent or milder than expected when compared to Bethlem myopathy. Creatine kinase levels were normal to mildly elevated. Muscle biopsies demonstrated necrosis, fiber size variation, fiber splitting, internal nuclei, and increased fat and connective tissue. *COL6A1* and *COL6A2* heterozygous missense mutations were identified in the three families.

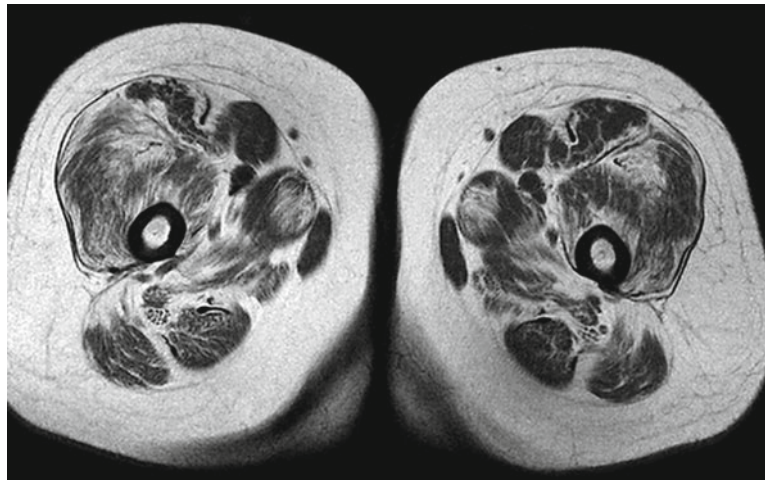

Fig. 12.2 Transverse T1 weighted images through the thigh muscles in a 7 year old girl with a collagen VI – related myopathy. Note the diffuse involvement with relative sparing of the sartorius and gracilis muscles. There is a rim of abnormal signal at the periphery of each muscle and relative sparing of the central part. There is prominent increase signal intensity in the central part of the rectus femoris (Courtesy of C.G. Bönnemann and J. Dastigir, NINDS/NIH, Bethesda, MD, USA)

12.1.1.4 Autosomal Recessive Myosclerosis Myopathy

Autosomal recessive myosclerosis myopathy has recently been described as a disorder of collagen VI [49]. Merlini et al. reported two siblings of consanguineous parents, characterised by Achilles tendon contractures and difficulty walking in early childhood, progressing to multi-joint contractures and the appearance of slender, 'woody', muscles [8]. The creatine kinase levels were elevated to twice normal. A homozygous nonsense COL6A2 mutation was found in the two siblings, which caused a disruption of collagen VI synthesis in cultured fibroblasts and discontinuous collagen VI staining in the basal lamina of myofibers [49]. The mutation affected the extreme C-terminus of the COL6A2 chain and allowed for the truncated COL6A2 chain to assemble with COL6A1 and A3 chains to form the triple helical monomers. Despite subsequent dimerization, higher assembly into tetramers was impaired [49].

12.1.2 Laboratory Findings

Creatine kinase is typically normal or elevated up to five times above the normal level. Creatine kinase levels may normalize over time. Electromyography generally demonstrates myopathic changes with short-duration, small amplitude action potentials and an increased proportion of polyphasic potentials. However, the EMG may be normal or demonstrate a neurogenic pattern with prolonged insertional activity and reduced number of high-amplitude polyphasic motor unit potentials. Muscle magnetic resonance imaging (MRI) has emerged as an increasingly valuable diagnostic investigation for patients with suspected collagen VI-related disease, especially in the milder cases where there may be greater diagnostic difficulty. The thighs demonstrate diffuse involvement with relative sparing of the sartorius, gracilis and adductor longus muscles [45–47] (Fig. 12.2). There is markedly increased signal at the periphery of the involved muscles with relative sparing of the central part of the muscle. Particularly in Bethlem myopathy there is increased signal in the anterior middle of the rectus femoris muscle centered around the central fascia, termed the "central shadow" [6, 47]. MRI of the calf muscles demonstrates diffuse increased signal involving predominantly the soleus and grastrocnemius muscles with relative sparing of the tibialis anterior and popliteus muscles [45, 47].

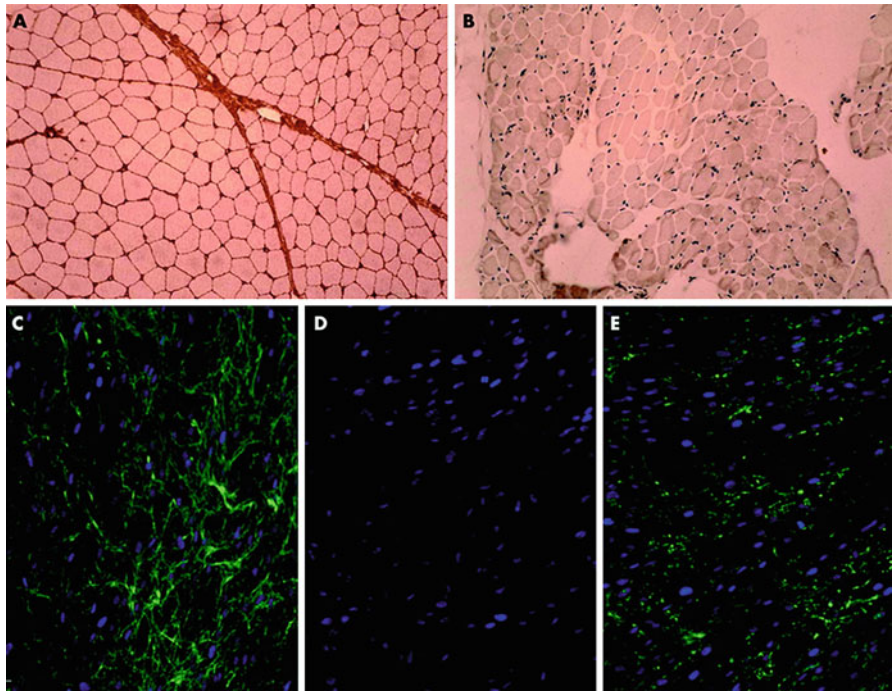

Fig. 12.3 Immunolabelling of collagen VI in skeletal muscle (panels **A** and **B**) and cultured dermal fibroblasts (panels **C** to **E**). Collagen VI is virtually absent in basement membrane surrounding muscle fibres of a UCMD patient with a homozygous splice mutation causing in-frame deletion in Nterminal triple helical domain of a3(VI) (**B**) when compared with a normal control (**A**). Fibroblasts from an unaffected control individual (**C**), the UCMD patient described above (**D**), and a BM patient with a heterozygous missense mutation in the N2 domain of a3(VI) (**E**) were grown in the presence of 50mg/ml L-ascorbic acid phosphate for five days after confluency. When stained with a collagen VI specific antibody (MAB3303, Chemicon) collagen VI is not detectable in the extracellular matrix of the UCMD patient and appears reduced and less organised in the BM patient (**E**) when compared with the normal control (**C**)

Muscle biopsy is of variable use in the diagnosis of these conditions. In UCMD muscle biopsy commonly shows dystrophic features with degeneration and regeneration and replacement of muscle with fat and fibrous connective tissue. Collagen VI immunolabeling from the endomysium and basal lamina ranges from absent to moderately or markedly reduced, but may be normal around capillaries [30] (Fig. 12.3b). If muscle is not available for collagen immunolabeling, loss of collagen VI in dermal fibroblast cultures may be a useful adjunct to the diagnosis [34]. In Bethlem myopathy the muscle biopsy typically reveals myopathic or dystrophic changes. Collagen VI immunolabeling of muscle is often normal or shows only subtle alterations in Bethlem cases, though, the immunofluorescent analysis of collagen VI in dermal fibroblasts may be predictive of Bethlem myopathy [28] (Fig. 12.3c–e). Confirmation of the diagnosis relies on molecular genetic testing. Molecular genetic testing using genomic DNA derived from peripheral blood samples of the three collagen VI genes detected putative mutations in 66 % of individuals clinically classified as having Bethlem myopathy, 56 % of individuals with Bethlem myopathy with an unusually severe phenotype and 79 % of individuals with Ullrich CMD [43]. The proportion of Bethlem myopathy and Ullrich CMD cases attributed to *COL6A1* and *COL6A2* mutations is roughly equivalent, at 38 % and 44 % respectively. Mutations in *COL6A3* however are less common at only 18 % of the total. Single amino acid substitutions

disrupting the Gly-Xaa-Yaa motif of the highly conserved triple helical domain of any of the three *COL6A* genes [35, 43, 44, 58, 64] constitute the most frequent pathogenic mechanism at 28 % of the total pathogenic variants the three collagen VI genes. Depending on their location, these mutations appear to either interfere with intracellular chain assembly, or, following successful secretion, to cause kinking of the tetramers, thus affecting extracellular microfibril formation [41]. Mutations that introduce premature termination codons (by splice site mutation, and out of frame deletions/insertions) form the second most frequent group, and truncate the protein, and some have been shown to result in absence of collagen VI because of nonsense-mediated mRNA decay [76]. Dominant heterozygously occurring splice mutations leading to in-frame exonic deletions as well as in-frame genomic deletions preserve a unique cysteine important for dimer formation, allowing secretion of abnormal tetramers with a consequent dominant-negative effect on microfibrillar assembly [2, 55]. Mutations which induce exon skipping occur at around 27 % of the total [40, 43, 44, 55, 59] and other splice-site mutations that cause small in-frame deletions or insertions have been reported. Large genomic deletions appear to be rare [43, 44, 71]. Given the high number of nonsynonymous polymorphic amino acid changes described for the collagen VI genes, it is difficult to be sure about the pathogenicity of missense mutations other than glycine substitutions within the triple helical domain. In recessive UCMD, a large number of mutations appear to result in premature termination codons with consequent nonsense-mediated mRNA decay. Splice mutations leading to in-frame exonic deletions as well as in-frame genomic deletions form another common mutation type in UCMD [2, 15, 32, 43]. Some genotype phenotype correlations have been made when an analysis of a cohort of 42 collagen VI-related myopathy patients with an early onset (under 2 years of age) by Brinas et al. showed that recessive mutations causing premature termination codons led to the most severe phenotypes (ambulation never achieved), whereas dominant de novo in-frame exon skipping and glycine missense mutations were identified in patients of the moderate-progressive group (loss of ambulation) [10]. Highly similar heterozygous genomic deletions were identified in a deletion prone region of *COL6A1* in two patients, one with a BM and one with a Ullrich CMD phenotype [60]. A novel type of mutation underlying recessively inherited UCMD recently emerged when 2 Ullrich CMD families were reported with large genomic deletions on chromosome 21 [21]. In addition to this, a deep intronic deletion was identified acting recessively in a BM patient by CGH array [7], although large or whole gene deletions appear to be rare in collagen VI-related disorders.

12.1.3 Biochemical Defect and Pathogenesis

Most muscular dystrophies arise as a result of mutations in genes which encode proteins that are part of the dystrophin-glycoprotein complex (DGC), a large oligomeric complex of proteins and glycoproteins of the sarcolemma that are critical to the stability of muscle fiber membranes and to the linking of the actin cytoskeleton with the extracellular matrix. However, some are due to mutations in genes encoding other sarcolemmal proteins and also those of the layer of extracellular matrix material that coats skeletal muscle fibres known as the basement membrane. Whereas the basement membrane was once considered a passive structure that merely provided mechanical support to the myofibre, new roles in muscle regeneration, myogenesis, synaptogenesis and signaling suggest the basement membrane is far more important than once thought [63]. Mutations in genes that encode proteins that do not directly link the DGC with the extracellular matrix (ECM) also cause muscular dystrophy, such as the collagen VI genes *COL6A1*, *COL6A2* and *COL6A3*. Whilst the pathogenic mechanism of the collagen VI-related diseases remains unknown, absence of the collagen VI microfibrils from the area adjacent to the basal lamina has been reported in UCMD patients [32, 33], suggesting a role for collagen VI in anchoring the basement membrane to the rest of the ECM. This

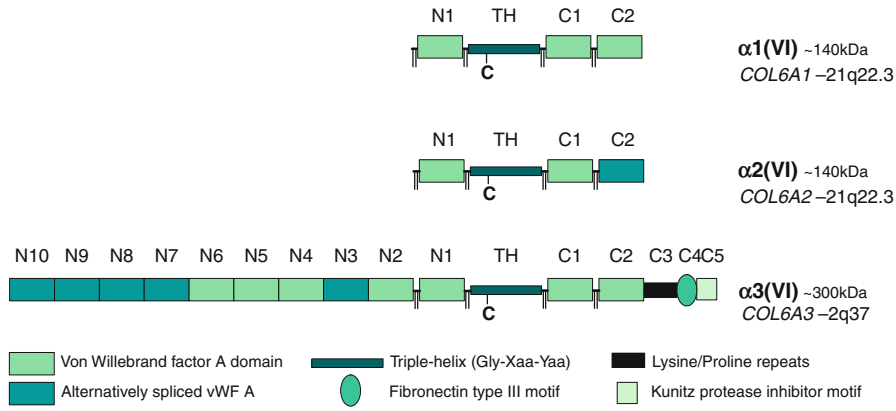

Fig. 12.4 Schematic representation of the three collagen VI polypeptide chains encoded by COL6A1-3.C represents the crucial cysteine residue required for dimerisation of the triple helical monomer (Modified from Lampe and Bushby [43. http://jmg.bmj.com/content/42/9/673.full?sid=3fee0735-53e2-4e9e-b6a6-f8d32452e26c)

lack of collagen VI at the sarcolemma can perhaps be explained by a reduced binding affinity of mutant collagen VI for the ECM of cultured myotubes [37] and fibroblasts [38]. Collagen VI appears to be crucial for the correct organisation of fibronectin in the ECM in fibroblast cells [62], suggesting that without this anchorage, the ECM tends toward disorganisation. Collagen VI is composed of three different peptide chains: α1(VI), α2(VI) (both 140 kd in size), and α3(VI) (260–300 kd in size) [18] (Fig. 12.3). The α1(VI) and α2(VI) chains are encoded by two genes *(COL6A1* and *COL6A2* respectively) situated head-to-tail on chromosome 21q22.3 [27] and separated by 150 kb of genomic DNA, whereas *COL6A3*, the gene for the α3(VI) chain, maps to chromosome 2q37 [75]. All three chains contain a central short triple helical domain of 335–336 amino acids with repeating Gly-Xaa-Yaa sequences flanked by large N- and C- terminal globular domains consisting of motifs of approximately 200 amino acids each that are homologous to von Willebrand factor (vWF) type A domains [13] (Fig. 12.4). Intron-exon boundaries are conserved with chicken collagen VI [72], suggesting that *COL6A1* and *COL6A2* genes arose by gene duplication before the divergence of the mammalian and reptilian lineages. Whilst collagen VI is ubiquitously expressed in adult human tissues [53], it exhibits a specific temporal and spatial pattern of expression during mouse development [16]. It is suggested that it is muscle interstitial fibroblasts, rather than cells of a myogenic lineage, that contribute collagen VI to the basement membrane surrounding the myofibre [77] and activation of a *COL6A1* enhancer element of connective tissue is the result of induction by secreted factors from myogenic cells [9]. Association of the three genetically distinct subunits, α1(VI), α2(VI), and α3(VI), to form a triple helical monomer is followed by staggered assembly into disulfide-bonded antiparallel dimers, which then align to form tetramers, also stabilized by disulfide bonds. Outside of the cell, tetramers, the secreted form of collagen VI, associate end to end to form the characteristic beaded microfibrils [18, 22, 42].

Investigation of collagen VI deficient mice developed our understanding of the collagen VI-related muscular dystrophies in a way that was not expected. Unlike patients with complete collagen VI deficiency presenting as severe UCMD, these mice had a very mild neuromuscular phenotype [5]. Despite the absence of clear signs of gross phenotypic abnormalities and or any impaired muscular function, muscle histology showed signs of myopathy including necrosis, phagocytosis and variation in fibre type diameter. Investigating the causative pathophysiological defects, it was shown that *Col6a1*$^{-/-}$

muscle displays a lack of contractile strength associated with ultrastructural alterations of the sarcoplasmic reticulum (SR) and mitochondria [31]. Further analysis of the *Col6a1*$^{-/-}$ null mouse implicated a dysfunction of the mitochondrial permeability transition pore (PTP) [1, 31, 49]. However, Hicks et al demonstrated that PTP dysregulation may not be specific to the collagen VI defect and showed that further work is needed on the relationship of PTP dysregulation with UCMD pathology [29]. Recently, *Col6a1*$^{-/-}$ null mouse muscle has been shown to be deficient in the autophagic process, suggesting a mechanistic link between mitochondrial dysfunction and muscle degeneration [23]. Forced activation of autophagy ameliorates the phenotype in the mouse model, providing a therapeutic target for UCMD.

The zebrafish model has also been used to consider the role of PTP dysregulation in collagen VI-related disorders. Morphants in which *col6a1* and *col6a3* genes had been knocked down by a morpholino approach displayed increased levels of apoptosis, in addition to impaired muscle development [67].

12.1.4 Treatment

In 2009 the International Standard of Care Committee for Congenital Muscular Dystrophy was established and developed the Consensus Statement on Standard of Care for Congenital Muscular dystrophy focusing on care issues such as neurological, pulmonary, orthopedic/rehabilitation, gastroenterology/nutrition, cardiology and palliative care interventions [74]. The mainstay of treatment for Collagen VI – related myopathies includes optimization of respiratory management with early implementation of noninvasive ventilation for nocturnal respiratory insufficiency. Further, careful orthopedic management with stretching, bracing, and selective surgical management of contractures and scoliosis can maintain and improve function. These interventions have improved the quality of life dramatically and have extended life expectancy [52, 65].

Currently there are no drug therapeutic interventions. However, knowledge of the molecular pathogenesis of the collagen VI–related myopathies has helped identify molecular and biochemical pathways that are now being explored as potential treatments, including anti-apoptotic, anti-fibrotic and gene/RNA therapies. Cyclosporin A (CsA) is a potent inhibitor of the mitochondrial PTP. In a study of five patients with collagen VI mutations treated with CsA for 1 month showed decreased apoptosis and increased stability of the mitochondrial PTP in fresh biopsies, although strength improvement in this limited study was not recorded [48]. The myopathic phenotype of the mouse and the mitochondrial pathology of UCMD patient myoblasts could be alleviated by the use of CsA which is also the permeability transition pore inhibitor [1]. Anti-apoptotic agents with less potential long-term toxicity have also been investigated. The cyclophin D inhibitor, D-MeAla3-EtVal4-cyclosporin (Debio 025) was developed from cyclosporine A by substituting Sar in position 3 and MeLeu in position 4 with D-MeAla and EtVal respectively [25]. Debio 025 does not display affinity for calcineurin, thus avoiding the immunosuppressive and toxic side effects of cyclosporine A. Tiepolo et al. demonstrated that Debio 025 also desensitized the PTP and decreased muscle fiber apoptosis in the diaphragm of *COL6a1* −/− mice [68]. Consistent with cyclophilin D as the presumed target of Debio 025, the disease was also ameliorated in a collagen VI/cyclophilin D double knockout mouse model [54].

Similarly, the antiapoptotic agent omigapil, will be investigated as a potential therapeutic agent for collagen VI–related disorders. Omigapil (SNT-201317, SNT-317, TCH346, and CGP3466) is a chemical derivative of selegiline but does not inhibit monoamine oxidase B (MAO-B), marginally inhibits MAO-A, and weakly interacts with α1-adrenergic, 5-HT2 and histamine-1 receptors [39]. Omigapil directly binds to GAPDH and inhibits the formation of the GAPDH-Siah1 complex thereby inhibiting the GAPDH-Siah1-CBP/p300 pathway-mediated apoptosis [12]. Erb et al showed that Omigapil

inhibited apoptosis in muscle of the laminin – 211 deficient (*dy/dy*) mouse model and ameliorated weight loss, improved kyphosis, motor function, and life span [19]. In the Col6a1$^{-/-}$ mouse model of collagen VI deficient – related myopathies (including UCMD), Omigapil has been demonstrated to inhibit apoptosis in the diaphragm and reduced mitochondrial defects in muscle (personal communication).

Strategies geared at ameliorating the fibrotic process also have been examined. Progressive fibrosis is a significant and common pathologic finding in collagen VI – related myopathy muscle biopsies, as it is in other congenital muscular dystrophies, and likely significantly contributes to the disease burden. Expression of transforming growth factor (TGF)-β, an inhibitor of muscle regeneration and a promoter of fibrosis, is elevated in muscle of laminin-α_2–deficient congenital muscular dystrophy patients (MDC1A), suggesting that interference with TGF-β would constitute a potential therapeutic target in this condition [3]. Angiotensin II promotes TGF-β mRNA expression, whereas the angiotensin II type 1 receptor antagonist losartan has been shown to attenuate TGF-β activation and improve regeneration and pathology in Marfan syndrome (extracellular matrix protein fibrillin-1 dysfunction) and dystrophin-deficient mouse models [14], in which TGF-β overactivity plays a role. Further, losartan treatment in laminin-α_2–deficient mouse models decreases serum TGF-β1 level and reduces downstream phosphorylated Smad2/3 proteins. There is improvement in muscle strength and amelioration of fibrosis [17].

The prevalence of nonsense-mediated mRNA decay in UCMD pathogenesis could be exploited for therapeutic gain. siRNA induced inhibition of hSMG-1, a critical kinase in the human nonsense- mediated mRNA decay pathway, resulted in an increase in collagen VI A2 mRNA in a patient with a homozygous COL6A2 mutation [69]. The obvious disadvantage to this sort of therapy would be the deleterious effect to cells which have lost the protective function of nonsense-mediated mRNA decay, although these fibroblasts have a normal growth rate and DNA damage response [70]. Whether collagen VI function was increased is unknown, but monomer assembly and export of the tetramer was possible [70]. In cases of dominantly inherited UCMD, where the mutated allele is exerting a strong, dominant negative force, siRNA mediated allele specific silencing has been proposed as a potential therapeutic approach. Targeted siRNAs were able to knock down *COL6A3* mutant transcript in patient fibroblast cells, improving cellular deposition of collagen VI [77].

12.1.5 Genetic Counseling

Bethlem myopathy is inherited in mostly autosomal dominant manner but some individuals with recessive inheritance have been reported [20, 24]. Ullrich congenital muscular dystrophy can be inherited in an autosomal recessive or an autosomal dominant manner. Most individuals with Bethlem myopathy are heterozygous for a *COL6A1*, *COL6A2*, or *COL6A3* mutation and are symptomatic. Parents of individuals with autosomal recessive Ullrich CMD are usually heterozygous for a *COL6A1*, *COL6A2*, or *COL6A3* mutation but do not appear to manifest any related symptoms. Individuals with dominantly inherited Bethlem myopathy are heterozygous for a *COL6A1*, *COL6A2*, or *COL6A3* mutation and are symptomatic. However, careful clinical examination may be necessary to identify findings diagnostic of Bethlem myopathy in minimally symptomatic parents of individuals with Bethlem myopathy [56]. Anticipation is not observed.

12.1.6 Related Disorders

As has been described above, mutations in the collagen VI genes cause a spectrum of myopathies with considerable phenotypic variability [43]. Bethlem myopathy [35] and UCMD [11] form either end of this phenotypic spectrum. Across the phenotypic spectrum, therefore the major differential diagnoses will also vary. For example in patients presenting in the congenital

period, the major differential diagnosis will be other forms of congenital muscular dystrophy and myopathies. In an LGMD presentation, the differential will include the defined LGMD subtypes. For the classical Bethlem presentation, the major differential diagnosis will be Emery-Dreifuss muscular dystrophy, and connective tissue disorders.

12.1.6.1 Limb-Girdle Muscular Dystrophy

Patients with proximal muscle weakness and minimal or no contractures can resemble a limb girdle muscular dystrophy phenotype. Immunohistochemical testing (i.e., western blotting and immunohistochemistry) performed on muscle biopsy and/or molecular genetic testing can help to establish the diagnosis of some LGMD subtypes. Further, muscle imaging can be helpful by recognizing LGMD subtype typical muscle pattern involvement.

12.1.6.2 Congenital Muscular Dystrophy

In an infant or a young child with moderate to severe hypotonia, weakness and joint contractures other forms of congenital muscular dystrophies must be considered. These include the CMD subtypes merosin – deficient MDC1a and the α-dystroglycanopathies, including Walker-Warburg syndrome, muscle-eye-brain disease, Fukuyama congenital muscular dystrophy, MDC1C *(FKRP* mutations) and MDC1D (*LARGE* mutations). In these patients however, the serum creatine kinase concentrations are much higher than those seen in UCMD. In addition, brain MRI may show abnormal white matter T2 – weighted hyperintensity or structural abnormalities. Further, in contrast to UCMD and BM, other CMD subtypes may have cognitive impairment. Patients with *SEPN1* – related myopathies (formally rigid spine muscular dystrophy) may also present similarly with a rigid spine but patients tend to have more neck and upper extremity weakness and less elbow and knee contracture involvement. Biochemical testing (i.e., western blotting and immunohistochemistry) performed on the muscle biopsy and molecular genetic testing can help to establish the diagnosis of some the CMD subtypes.

12.1.6.3 Central Core Disease

Similar to patients with collagen VI-related myopathies most patients with central core disease (CCD), resulting from *RYR1* mutations, have mild disease with symmetric proximal muscle weakness and variable involvement of facial and neck muscles. Motor development is usually delayed and in more severe disease can be early in onset with hypotonia, spinal deformities, joint laxity/contractures, and early respiratory insufficiency. Muscle biopsies from patients with collagen VI-related myopathies can also include minicore-like lesions or congenital fiber type 1 disproportion resembling those patients with *RYR1*- associated multiminicore disease or congenital fiber type 1 disproportion. Muscle imaging can be particularly helpful by recognizing the *RYR1* typical muscle pattern involvement [36].

12.1.6.4 Emery-Dreifuss Muscular Dystrophy

In patients with contractures as a prominent feature, Emery-Dreifuss muscular dystrophy (EDMD) should be a consideration. EDMD is caused by mutations in *LMNA*, *EMD* and *FHL1*. EDMD is associated with serious cardiac involvement differentiating it from collagen VI – related myopathies in which primary cardiac involvement is not seen. Mutations in *LMNA* may also present as a CMD subtype with significantly more neck weakness and early rigidity of the spine [61]. Due to the high risk of cardiac complications associated with Emery Dreifuss Muscular dystrophy, these diagnoses should be carefully considered in patients with a contractural phenotype.

12.1.6.5 Ehlers-Danlos Syndrome

In patients with joint hypermobility as the predominant feature, Ehlers-Danlos syndrome (EDS) needs to be considered. Typically, patients with EDS classical form (*COL5A1*/*COL5A2* mutations) and hypermobility forms (*TNXB* mutations), weakness and contractures will be milder than Bethlem myopathy. Further, patients

with EDS may have hyperelasticity of the skin which is not seen in collagen VI-related myopathies. In a child with mild to moderate weakness and significant joint laxity, EDS type VI kyphoscoliosis form (*PLOD1*, *CHST14*, and *FKBP14* mutations), in particular should be considered.

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Mouse Models in Tendon and Ligament Research

13

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Abstract

Mutant mouse models are valuable resources for the study of tendon and ligament biology. Many mutant mouse models are used because their manifested phenotypes mimic clinical pathobiology for several heritable disorders, such as Ehlers-Danlos Syndrome and Osteogenesis Imperfecta. Moreover, these models are helpful for discerning roles of specific genes in the development, maturation, and repair of musculoskeletal tissues. There are several categories of genes with essential roles in the synthesis and maintenance of tendon and ligament structures. The form and function of these tissues depend highly upon fibril-forming collagens, the primary extracellular macromolecules of tendons and ligaments. Models for these fibril-forming collagens, as well as for regulatory molecules like FACITs and SLRPs, are important for studying fibril assembly, growth, and maturation. Additionally, mouse models for growth factors and transcription factors are useful for defining regulation of cell proliferation, cell differentiation, and cues that stimulate matrix synthesis. Models for membrane-bound proteins assess the roles of cell-cell communication and cell-matrix interaction. In some cases, special considerations need to be given to spatio-temporal control of a gene in a model. Thus, conditional and inducible mouse models allow for specific regulation of genes of interest. Advances in mouse models have provided valuable tools for gaining insight into the form and function of tendons and ligaments.

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Fibril-forming collagens • *oim* mouse model • *cho* mouse model • Chondrodysplasia • *Ppib* • *Crtap* • or *Lepre* null mice • *Col14a1*$^{-/-}$ mouse model • Collagen XIV • Mouse model for mutated *Col12a1* • *Scleraxis* transgenic model mice • *Scx*-null (*Scx*$^{-/-}$) mice • Osteogenesis Imperfecta • Haploinsufficient *Col3a1*$^{+/-}$ and *Col5a1*$^{+/-}$ mice

13.1 Introduction

Analyses of connective tissue biology have benefited from the use of animal models. These models have been valuable for dissecting out the mechanisms involved in development, maturation, and repair. Mouse models will continue to be important in defining roles, relationships, and interactions involving proteins critical to connective tissue structure and function as well as pathophysiology. This chapter provides an overview of different mouse models useful in tendon and ligament research. This includes models addressing the roles of (1) fibril-forming collagens as well as interacting molecules regulating tendon fibrillogenesis; (2) growth factors and their receptors; (3) transcription factors and (4) cell surface receptors. In addition, application of conditional and inducible mouse models will be outlined and prospects for this technology will be discussed. A model for tendon and ligament collagen fibrillogenesis, fibril assembly, and maturation is presented in Fig. 13.1. In this model, genes and their respective proteins which have been implicated in regulation of tendon and ligament pathobiology are highlighted. The continued innovative use of mouse models will be essential to continue progress in the elucidation of tendon and ligament biology as well as pathophysiology. In addition, mouse models will provide a foundation for preclinical studies necessary for continued therapeutic advances in treatment of disorders of tendons and ligaments resulting from trauma, injury, and overuse as well as genetic diseases involving connective tissues like Osteogenesis Imperfecta and Ehlers-Danlos Syndrome.

13.2 Mouse Models for Structural and Fibril-Forming Collagens and Related Molecules

Mouse models that alter the expression of collagens and related regulatory molecules have been important in elucidating the roles of these molecules in collagen synthesis, processing, assembly, and organization, as well as structural homeostasis of the formed tissue. This section will provide an overview of mouse models for fibril-forming collagens I, V, and XI, as well as enzymes such as prolyl 3-hydroxylase 1 and cyclophilin B that affect their processing. Models will be described for FACIT (Fibril Associated Collagens with Interrupted Triple helices) collagens XII and XIV as will models for regulatory small leucine rich proteoglycans (SLRPs). Finally, within this section, models for the ubiquitous beaded filament-forming collagen VI and for glycoprotein fibrillin will be discussed.

13.3 Fibril-Forming Collagens

Fibril-forming collagens I, II, III, V, and XI have been found in tendons and in ligaments. Collagens I, II and III are the most abundant proteins in the vertebrate body and are the bulk components of all collagen fibrils. Within tendons and ligaments, collagen I is the predominant collagen. Collagen III is found in greatest abundance during embryonic development; however, it is detectable in mature tendons (5 % collagen composition) and ligaments (10 % collagen composition) [1, 2]. Collagen II is generally found within the fibrocartilaginous zone of the enthesis site for both tendons and ligaments [3, 4]. Though collagens V

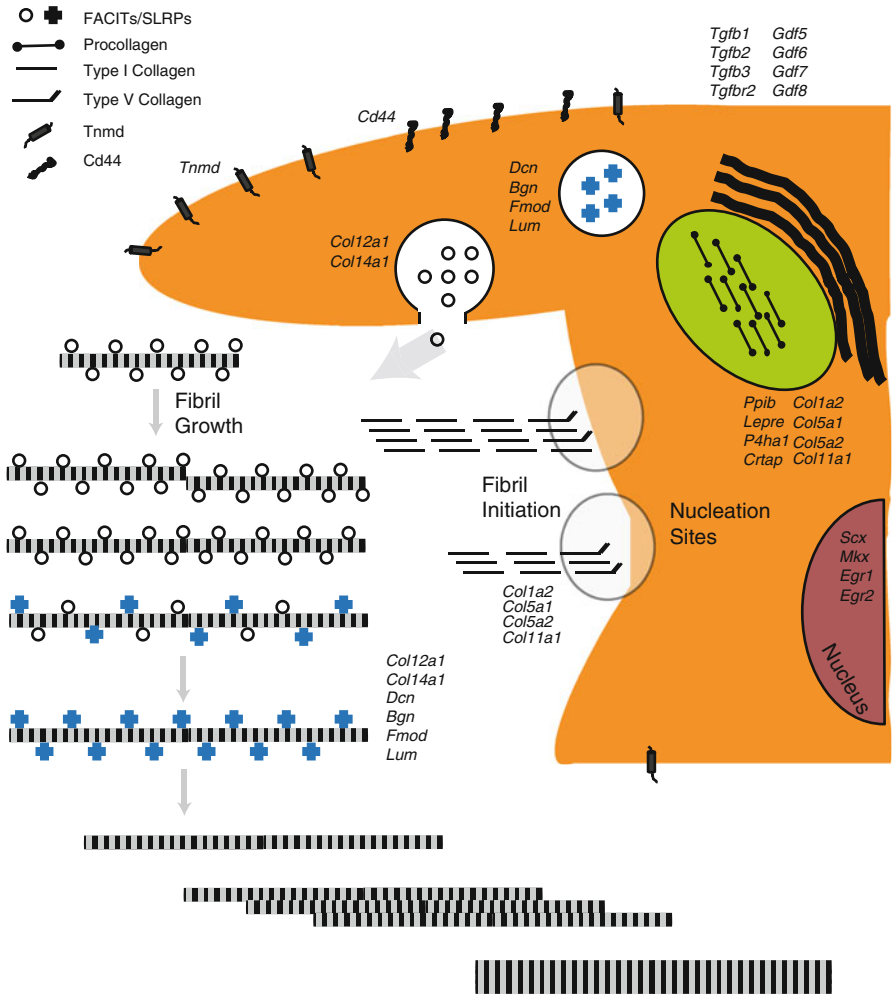

Fig. 13.1 Mouse models utility in defining a gene's role in collagen fibrillogenesis. Many mouse models have been developed that can be used to define the roles in collagen fibrillogenesis of specific genes individually or in combination. In this illustration, collagen fibrillogenesis is depicted. Genes for mouse models are described are listed within the illustration at those points in collagen fibrillogenesis where their involvement has been shown to have a significant effect

and XI are quantitatively minor collagens, they are found co-assembled with collagens I, II and III, and they are essential to the regulation of fibril assembly.

Several fibril-forming collagen mouse models exist. The essential roles of fibril-forming collagens during development and maturation are emphasized in a survey of these mutant models. In most cases for fibril-forming collagens, homozygous null mutations for these genes result in embryonic lethal phenotypes. The first collagen null mouse, the Mov13 mouse, contained a single Moloney murine leukaemia virus (M–MuLV) proviral copy in the first intron of the α1(I) collagen gene (*Col1a1*) that was recessive lethal and interfered with the synthesis of stable α1(I) collagen messenger RNA [5–7]. The mutant mouse for *Col1a2* is a model for Osteogenesis Imperfecta and is called the *oim* mouse model [8]. *Col2a1* null mice demonstrated major pathological phenotypes for articular cartilage, the notochord, and in skeletal development [9–11] while most $Col3a1^{-/-}$ mice die prior to birth [12–14]. However, haploinsufficient $Col3a1^{+/-}$ mice

demonstrated an aortic dissection/vascular phenotype comparable to that in Ehlers-Danlos syndrome, type IV. Mutant models also exist for *Col5a1* as a null mutant and for *Col5a2* as a dominant negative model [15–18]. *Col5a1*$^{-/-}$ mice are also embryonic lethal and thus could only initially be studied as heterozygous *Col5a1*$^{+/-}$ haploinsufficient mice that are models for the classic form of Ehlers-Danlos syndrome. Likewise, a mutant mouse has been generated for *Col11a1*; it is a model for chondrodysplasia and is called the *cho* mouse model [19]. Homozygous *Col11a1*$^{-/-}$ mice typically die soon after birth [19]. Conditional and inducible mouse model strategies, however, have made it possible to study null mutations in a temporal-spatial manner.

Osteogenesis Imperfecta is a heritable connective tissue disorder linked to mutations in pro(α1) or pro(α2) chains of collagen I for a majority of patients and by recessive mutations in molecular chaperones and processing enzymes for a small percentage of patients [20, 21]. Models for recessive mutations of processing enzymes are discussed in the next sub-section. The primary clinical features include short stature, multiple fractures, skeletal deformities, osteopenia, and joint laxity [20]. In mice, a natural mutation was discovered in *Col1a2* in a mouse line at Jackson Laboratories; this mouse line was termed the *osteogenesis imperfecta murine* line, or *oim* [8]. Homozygous *oim* mice have several severe phenotypes at birth, including hemorrhages into joint cavities and around the scapulae, visible long bone fractures, and subluxation of both forepaws [8]. In mature mice, several healing fractures and long bone deformities are evident [8]. Without *Col1a2*, a general osteopenia occurs as cortical bone thins with decreased numbers of osteocytes and disorganized cortical lamellae [8]. Phenotypes were noted for skeletal muscle, tail tendons, and aortas of *oim* mice [22–25]. The tail tendons in these *oim* mutant mice demonstrated reduced tensile strength and changes in structure [23, 24]. The average collagen fibril diameter in tail tendons of *oim* mutant mice was about half that of wild type tail tendons [24]. Moreover, *oim* tendons could not withstand the same levels of stress as wild type tendons; wild type tendons could be stretched with as much as twice as much tension as those of the *oim* mice [24]. While the D-period of the fibrils appeared indistinguishable, X-ray diffraction images of *oim* tail tendon collagen revealed no characteristic "triplet" lateral reflection, which is an indication of a loss of the typical lateral packing structure [23]. Thus, the typical triple helix structure is compromised and fibrils are more susceptible to degradation and enzymatic digestion, and thus they are less stable [23]. The lack of stability was demonstrated with possible compensation via increased cross-linking patterns in collagen fibrils in aortas of *oim* mice [25]. Analyses of the *oim* mouse have made significant contributions to the definition of the functional role of the collagen α2(I) chain in collagen fibril structure and function in musculoskeletal tissues.

Several mouse models exist for analyses of the regulatory/functional roles of collagen V. Collagen V is involved in nucleation of collagen fibril assembly [17, 18]. There are different isoforms with different functions including the alpha 1(V) homotrimer in interfacial regions [26] and the alpha 3 (V) isoform [27]. One well studied model is the *Col5a1*$^{-/-}$ mouse. The expression of the homozygous *Col5a1*$^{-/-}$ mutation is lethal at embryonic day 11 in mice with signs of cardiovascular insufficiency as a result of the mutation [17]. The homozygous *Col5a1*$^{-/-}$ mouse has a virtual absence of collagen fibrils in the presence of normal amounts of collagen I. The use of this model demonstrated a critical regulatory role for collagen V in the nucleation of collagen I assembly and initial fibril assembly [17].

Heterozygous *Col5a1*$^{+/-}$ mutants are haploinsufficient, synthesizing approximately half the normal collagen V [17]. The classic form of Ehlers Danlos Syndrome is a broad spectrum connective tissue disease. This form of the disease can be characterized as having mutations involving collagen V [28–30]. In the haploinsufficient EDS model collagen fibrils of the dermis were larger overall and generally irregularly shaped [17]. In addition, fewer fibrils were assembled with these data, consistent with dysfunctional regulation of nucleation of collagen

assembly and initial fibril formation. The phenotype of the *Col5a1*$^{+/-}$ mouse model is nearly identical to the clinical phenotype in the classic form of Ehlers-Danlos syndrome that comprises a broad spectrum of connective tissue defects including hyper-extensible and fragile skin [17]. Biomechanical analyses of the flexor digitorum longus (FDL) tendons of *Col5a1*$^{+/-}$ model demonstrated a smaller cross-sectional area and significantly reduced stiffness, when compared to wild type tendons, [18] consistent with the hypermobile joint phenotype. Fibril structure in *Col5a1*$^{+/-}$ tendons was altered [18]. Thus, similar to the dermis, results of structural and biomechanical analyses of testing of *Col5a1*$^{+/-}$ mutant flexor (FDL) tendons indicated increased elasticity. Recently a conditional mouse model for *Col5a1* was described [31]. This mouse model will permit targeting of the mutation to specific tissues and avoid the embryonic lethal phenotype in the traditional knock out model. Use of an inducible Cre will permit temporal analyses to dissect out specific functions at different times as in wound repair or development.

Collagen XI and collagen V are homologous in structure and function and should be considered different forms of the same collagen type. Both are regulatory fibril-forming collagens that regulate fibril assembly. A mutation for *Col11a1* was originally described as the *chondrodysplasia* (*cho*) mouse [19, 32]. Homozygous *Col11a1*$^{-/-}$ mutants died shortly after birth because weakness in the trachea could not support normal breathing [32]. The *Col11a1* mutation in mice is important in chondrogenesis and endochondral ossification; hyaline and growth plate cartilage are affected as with human fibrochondrogenesis [32, 33]. Because *Col11a1*$^{-/-}$ mouse mutants, also known as *cho/cho*, die shortly after birth, *cho/+* heterozygotes were studied. Articular cartilage from the knees of *cho/+* heterozygotes had notable degradation by 15 months as collagen II denatured [34]. In *cho/cho* mice tendons at 30 days old, fibrils had a heterogeneous distribution of diameters, including a subpopulation that were large and irregularly shaped, and fiber organization was disrupted [18]. This effect was more severe in combined mutant *Col5a1*$^{+/-}$ *Col11a1*$^{-/-}$ mice [18]. In *Col11a1*$^{+/-}$, *Col11a1*$^{-/-}$, and *Col5a1*$^{+/-}$ *Col11a1*$^{-/-}$ mice, there were reductions in the total fibril number assembled [18]. Reductions in tendon cross-sectional area and fibril numbers suggest that fewer fibrils are enucleated by tendon fibroblasts. Thus, findings from the *Col5a1*$^{+/-}$, *Col11a1*$^{-/-}$, *Col11a1*$^{+/-}$, and *Col5a1*$^{+/-}$ *Col11a1*$^{-/-}$ mice all suggest that there are coordinate roles for collagens V and XI in the regulation of fibril nucleation and initial assembly in early tendon development.

Fibril-forming collagens are essential to all extracellular matrices. Mutant mouse models are excellent resources for examining the role of each individual gene as well as combinations of genes in the tissue development. They serve as models to characterize human connective tissue diseases that result from heritable mutations. Table 13.1 lists those mouse models that have been useful for understanding mechanisms of disease for human connective tissue disorders.

13.4 Enzymes Affecting Collagen Processing

Several enzymes are essential to collagen processing. As collagen propeptides are synthesized, two unique amino acids – hydroxyproline and hydroxylysine – are formed through modification of amino acids by enzymatic hydroxylation of almost all prolyl- and some of the lysyl-residues in the Y-positions of the Gly-X-Y structure. These modified amino acids are important for downstream triple helix stability and glycosylation. In order for triple helix formation to occur after translation, a *cis* to *trans* isomerization of Gly-Pro- (of the Gly-X-Y) is necessary [35]. This is catalyzed by cyclophilins that act as peptidyl prolyl cis/trans-isomerases. Along with cyclophilin, prolyl-3-hydroxylase and cartilage-associated protein (Crtap) form a ternary complex with high chaperone activity in the endoplasmic reticulum that introduces a single 3-Hyp-residue at the C-terminal end of the triple helical domain of nascent fibrillar procollagens and allows for efficient catalytic isomerization of peptidyl-prolyl cis bonds by prolyl-4-hydroxylase

Table 13.1 Mouse models that parallel clinical manifestations of human genetic disorders

Null mutant mouse model	Human disorder	Shared phenotypes or clinical manifestations	References
Col1a2 (*oim*), *Pbib, Crtap, Lepre*	Osteogenesis Imperfecta	Short stature; multiple fractures; skeletal deformities; osteopenia; joint laxity; delayed skull ossification; osteoporosis; reduced aortic integrity; loose, thin skin	[8, 22, 24, 25, 37, 39, 41, 45]
Col5a1, Col5a2	Ehlers-Danlos syndrome	Skin hyperextensibility, articular hypermobility, tissue fragility, joint laxity, kyphoscoliosis, aortic rupture, cardiovascular insufficiency	[12–19, 30, 31]
Col6a1	Bethlem myopathy or Ullrich congenital muscular dystrophy	Myopathy including muscle fiber necrosis, phagocytosis, and weakened muscle fiber integrity; hyper-extensible and fragile skin; joint laxity	[81–84]
Col11a1 (*cho*)	Chondrodysplasia or Marshall/ Stickler syndrome	Midface hypoplasia, articular cartilage and growth plate fibrochondrogenesis, osteoarthritis	[18, 19, 30, 32–34]
Fbn1, Fbn2	Marfan syndrome	Cardiovascular complications, mild to moderate joint laxity, scoliosis, muscle weakness	[88–93]
Gdf5 n*bp*)	Hunter-Thompson or Grebe type chondrodysplasias, brachydactyly C	Altered bone lengths segment numbers in digits, ankylosis	[112, 114–118]

[35–38]. Mouse models for cyclophilin B (*CypB* or *Ppib*), cartilage associated protein (*Crtap*), and prolyl-3-hydroxylase (*P3h1 or Lepre*) have been described [37, 39–44].

Combinations of mutations in *Ppib*, *Crtap*, and *Lepre* can cause Osteogenesis Imperfecta (OI) in its recessive form [21]. Null mice models for these genes have been generated and the models demonstrate OI phenotypes. Mutations in these genes affect prolyl-3-hydroxylation of collagen. *Ppib*-null mice and *Lepre*-null mice both demonstrate kyphosis and severe osteopenia [41, 44]. *Crtap*-null mice had reduced levels of bone formation, as well as osteoporosis [45]. Moreover, analyses of collagen I demonstrated for all three null mice almost complete absence of 3-hydroxyproline in the peptide at Pro-986, though collagens from wild type and heterozygote littermates had an abundance of peptides containing hydroxyproline in that very position [41, 44, 45]. Still, for the null mice, known prolyl–4 hydroxylation sites at residues 981 and 987 were modified appropriately [41, 44, 45]. Because 3-hydroxylation of Pro-986 in collagen I is required for correct fibril formation, the majority of the collagen fibrils in the *Ppib*-null mice had larger diameters than wild type littermates; furthermore, because these genes act in the endoplasmic reticulum (ER), much of the collagen I was still subcellularly localized in the ER instead of being secreted [41, 45]. Tendons of *Lepre*-null mice exhibit severe ultrastructural defects such as decreased fibril diameters, longitudinal axial twists, fibril branching into smaller fibrils, lack cellular processes [44]. Like with OI, skin in the *Ppib*-null mice was loose and thin, contained less collagen I, and was biomechanically weaker [41]. Levels of P3h1 were reduced without CypB, most likely contributing to reduced levels of appropriate prolyl-3-hydroxylation of collagen [41]. As prolyl 3-hydroxylase is a component of the P3h1-Crtap-CypB complex, mice that are deficient in P3h1, or *Lepre*-null mice, demonstrate a similar phenotype as the *Ppib*-null mice; these mice have a delay of ossification in the skull with reduced levels of collagen in the bone and skin [44]. These three null mice models

closely resemble OI types VII and VIII that involve mutations to genes within the enzyme complex that promote the prolyl-3-hydroxylation of fibril-forming collagens [37, 40].

13.5 Regulatory Molecules: FACITs

Fibril-Associated Collagens with Interrupted Triple Helices (FACIT) interact with collagen fibril surfaces. These molecules affect the surface properties of fibrils as well as fibril packing. While FACIT collagen IX is primarily found interacting with collagen II and proteoglycans in cartilage, FACIT collagens XII and XIV have been found throughout musculoskeletal connective tissues, including tendons and ligaments at various times during development [46–50].

Collagen XIV is implicated in the regulation of tendon fibril growth in early tendon development; even in the postnatal mouse, expression of collagen XIV in the flexor digitorum longus (FDL) was greatest at 4 days old followed by a rapid reduction with maturity [46]. Targeted deletion of *Col14a1* in a *Col14a1*$^{-/-}$ mouse model demonstrated a premature entrance into the fibril growth stage in tendons, resulting in larger diameter fibrils in early developmental stages with a loss of associated fibroblast compartmentalization of fibrils, thus disrupting fiber formation [46]. Fiber organization appeared to improve with maturity; however, by 60 days of age, *Col14a1*$^{-/-}$ fibril cross-sectional diameters were once again larger [46]. The larger diameter *Col14a1*$^{-/-}$ fibril diameters at 4 days old is indicative that in some tissues like tendons FACIT collagen XIV serves as 'gate keeper' regulating transition into the fibril growth phase (Fig. 13.2). An additional role in fibril packing has been long suspected to be due to the large non-collagenous domain and its inter-fibrillar location. Control of fibril packing would influence lateral associations necessary for fibril growth. Collagen XIV, thus, temporarily stabilizes protofibrils to prevent the initiation of lateral fibril growth. Without regulation by collagen XIV, the resulting tendons can manage less maximum load and stress and are less stiff ahead of maturation [46]. However, after tendon maturation, the role of collagen XIV is less obvious as *Col14a1* expression diminishes in wild type FDL and biomechanical properties are similar in both wild type and *Col14a1*$^{-/-}$ mouse FDL at maturity [46]. Still, the importance of collagen XIV in modulating mechanical strength should be noted in light of findings in bovine medial collateral ligaments, where collagen XIV is found at high concentrations in the ligament-bone interface that receives a great deal of mechanical stress [49]. The enrichment of collagen XIV in regions of mechanical stress is clearly an aspect of FACIT biology that needs further study.

The role of FACIT collagen XII in tendon development has yet to be elucidated. However, evidence suggests that collagen XII is a stress response molecule directly influenced by stretch and shear stress. It was reported that the *Col12a1* gene could be directly stimulated by mechanical forces in fibroblasts [51, 52] and endothelial cells [53] as well as osteoblasts [54]. The mechanical strain response element is conserved in the first intron of the Col12a1 gene in chicken, human [55] and mice [54]. Two mouse models have been developed that might be used to explore the role of *Col12a1* in tendon development. The first mouse model contains dominant interference transgenic mutation of *Col12a1*; briefly, a truncated minigene construct driven by a *Col1a2* promoter causes severe disorganization of the ligamentous collagen bundles in the periodontal ligament, including the disruption of the parallel fiber alignment with a seemingly porous consistency [56]. In a second mouse model, a targeted mutation *Col12a1* was shown to affect osteoblast differentiation and alignment, cell-cell communication, and bone matrix formation [47]. Thus, work with the null mouse suggests that not only does *Col12a1* play a critical role in collagen organization, it is also necessary for maintenance of cell-cell and cell-matrix interactions that affect cell differentiation. Further utilization of these FACIT mouse models should improve our understanding of tendon and ligament development, as well as any roles in tissue strength, tissue homeostasis, and healing.

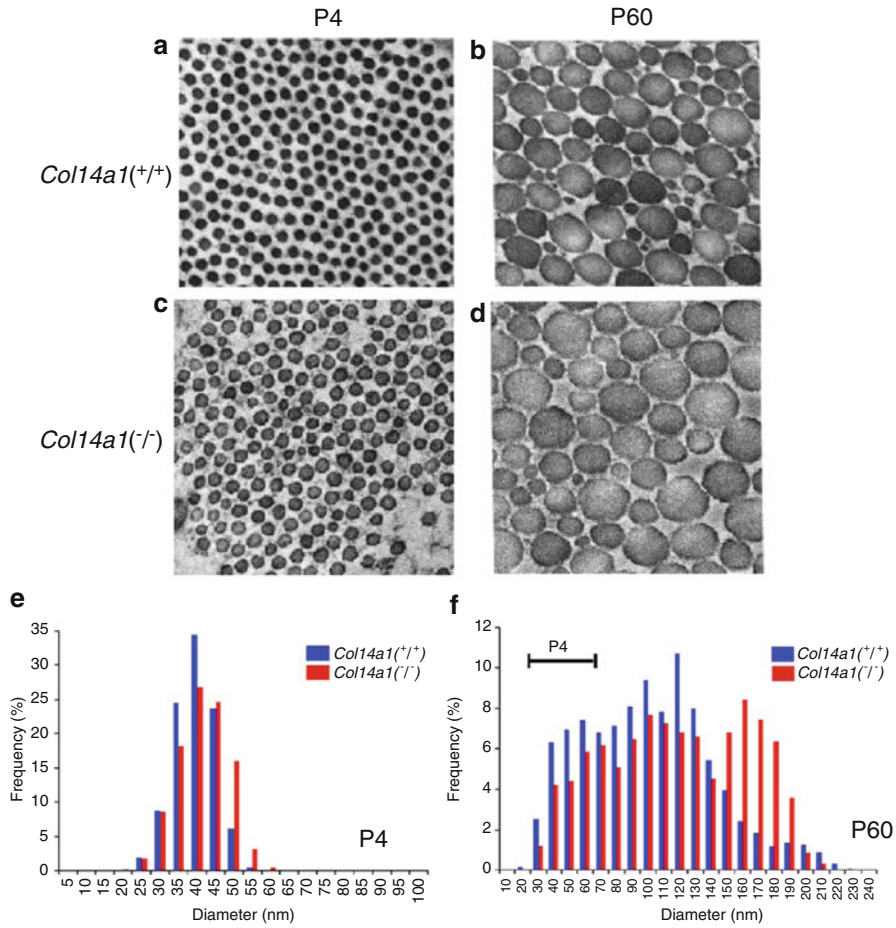

Fig. 13.2 Fibril growth is affected in *Col14a1*$^{-/-}$ tendons. Transmission electron micrographs of flexor digitorum longus (FDL) tendons were examined at postnatal day 4 (P4) and 60 (P60) for *Col14a1*$^{+/+}$ and *Col14a1*$^{-/-}$ mice. P4 wild type control fibrils (**a**) are more uniform in shape than P4 *Col14a1*$^{-/-}$ tendon fibrils (**c**) that are more irregularly shaped. An examination of each genotype's fibril diameters at P4 (**e**) indicates that wild type control fibrils are smaller than *Col14a1*$^{-/-}$ tendon fibrils. At P60 (**b**), mature fibrils of the wild type tendon are of variable size with small to larger cross-sectional profiles, while fewer smaller fibrils and more fibrils of larger diameter are seen amongst *Col14a1*$^{-/-}$ tendons (**d, f**). A comparison of fibril diameters expected at P4 versus P60 is noted with a bar (**f**). The bar in electron photomicrographs (**a–d**) represents 100 nm (This research was originally published in the Journal of Biological Chemistry (Ansorge HL et al. © the American Society for Biochemistry and Molecular Biology, [46]))

13.6 Regulatory Molecules: SLRPs

Once collagen protofibrils are assembled and deposited into the extracellular matrix of tendons and ligaments, further assembly involves linear and lateral growth of the preformed fibril intermediates. Beside FACIT collagens, another category of regulatory molecules called small leucine-rich proteoglycans (SLRPs) is crucial for regulation of linear and lateral fibril growth. Two classes of SLRPs are expressed throughout tendon growth and maturation: class I (decorin and biglycan) and class II (fibromodulin and lumican). The roles of these SLRPs in collagen fibril assembly and growth have been investigated using several mouse models. When the genes for

these molecules are specifically targeted as in single or compound SLRP deficient mice, their importance in regulation of linear and lateral fibril growth in tendons is demonstrated [57–60]. Generally, in tendons, decorin and fibromodulin are dominant in this regulation, and they can be modulated by biglycan and lumican, respectively [61, 62]. The lack of decorin, biglycan, or fibromodulin leads to disruptions in fibril growth, resulting in alterations in fibril diameters fibers, structural abnormalities, and biomechanical alterations in the tendon [61–63]. Moreover, additive effects are seen between classes when compound biglycan and fibromodulin deficiencies occur, that affect fibril diameters, alter tendon biomechanics, and even promote ectopic ossification within the tendon [57, 63, 64]. Unlike fibril-forming collagens, SLRPs regularly turnover and more easily allow for changes in expression to affect fibrillogenesis and tendon structure throughout development, maturation, and injury [65]. In the following paragraphs, specific examples where these mouse models were used to discern SLRP function will be presented.

A mouse model deficient in decorin demonstrates the critical role that decorin plays in regulation of collagen fibril assembly [66]. In *Dcn*$^{-/-}$ mice, cross-sectional fibril profiles were irregular versus the normal circular profiles; these became more evident with maturation [66]. Throughout postnatal development and into maturation, the fibrils had greater diameters in *Dcn*$^{-/-}$ mice, that is indicative of a lack of regulation of lateral fibril growth in the absence of decorin (Fig. 13.3) [66]. Biomechanical testing of *Dcn*$^{-/-}$ FDL tendons showed decreases in mechanical strength – maximum load and maximum stress – and stiffness occurred in mature tendons (150 days old) [66]. Biglycan content was analyzed in the *Dcn*$^{-/-}$ tendons; biglycan levels were increased, suggesting some functional compensation in regulation of fibril growth [66]. However, as biglycan expression decreases with maturation, absence of both class I SLRPs may be noted in the *Dcn*$^{-/-}$ mice. Thus, without *Dcn* expression in tendon development, regulation of fibril growth is affected. It has been shown that decorin and biglycan interact with the same collagen I site, but with differing affinities [67] and that biglycan can functionally compensate for decorin in in vitro fibrillogenesis assays [68].

In tendon, biglycan (*Bgn*) expression peaks early in postnatal development before decreasing with maturation [66, 69]. Biglycan also has been targeted to assess its musculoskeletal roles in null mice. Much of the current literature where *Bgn*$^{-/0}$ mice phenotypes are reported include changes to bones [70]. These changes include: decreased long bone length, decreased cortical bone thickness, decreased trabecular bone volume, reduced biomechanical bone strength, reduced mineralization, and reductions in mesenchymal stromal cell numbers within bone [70]. However, tendon phenotypes have been described for *Bgn*$^{-/0}$ mice; fibril cross-sectional diameters of quadriceps tendons at 90 days are smaller than wild type [64]. Moreover, menisci of mature *Bgn*$^{-/0}$ demonstrated ectopic ossification, and cruciate ligaments of exercised *Bgn*$^{-/0}$ mice were hypertrophic and/or torn [71]. When the *Bgn*$^{-/0}$ mutation is combined with a targeted fibromodulin (*Fmod*$^{-/-}$) mutation, notable defects and phenotypes develop (Fig. 13.4) [64, 71, 72]. Tendons of *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice were smaller in area, hypercellular with more stem/progenitors, and disorganized with gaps within collagen fibrils [72]. Recurrent, transient abnormal leg dragging and decreased flexibility typify the gait of *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice beginning at 3 weeks of age and persisting throughout life [64]. Ectopic ossification of *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ tendons was detectable around 3 months by radiograph, yet increased in severity with age; ossification was seen in menisci [64, 71]. Leg dragging and ectopic ossification were also accompanied by severe knee osteoarthritis with complete erosion of articular cartilage by 6 months [64]. Tendons of the compound mutants had increased numbers of small diameter fibrils resulting in a smaller mean diameter [64]. *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice tendons were mechanically less stiff and thus less stable and less supportive [64]. A mechanism for the phenotypic changes was proposed in these *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice; the lack of biomechanical strength caused

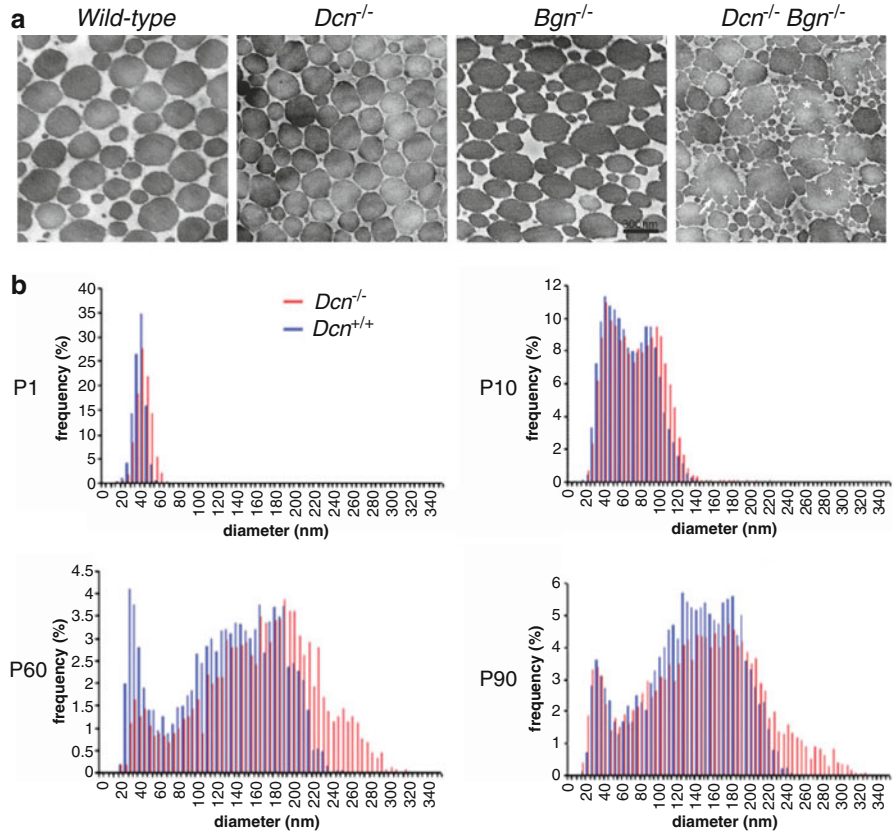

Fig. 13.3 Altered collagen fiber distributions in *Dcn*$^{-/-}$ mice. (**a**) An examination of wild type, *Bgn*$^{-/-}$, *Dcn*$^{-/-}$, and *Bgn*$^{-/-}$ *Dcn*$^{-/-}$ FDL tendon cross sections demonstrates changes in fibril diameter and shape in the absence of these SLRPs, particularly the synergistic results of a compound mutation. (**b**) For *Dcn*$^{-/-}$ tendons, specifically, at each stage of postnatal maturation, mutant mouse fibril diameters were larger than those of wild type tendons. P1 represents a period of fibril intermediate assembly. P10 and P60, respectively, represent the beginning and the end of lateral fibril growth. P90 represents the mature tendon (This figure was adapted from research originally published in the Journal of Cellular Biochemistry (Zhang G et al. © John Wiley & Sons, Inc., [66]))

by the loss of the two SLRPs results in joint instability and changes in gait, followed by osteoarthritis and ectopic ossification [64]. When *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice are exercised on a rotarod, the abundant ectopic ossification of tendons, compromised tendon strength, meniscal tears, and osteoarthritis lead to deficiencies in exercise [71]. Besides manifesting in the knee joint, osteoarthritis was demonstrated in the temporomandibular joint of *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice [73]. Deficiencies in biglycan affect tendon and ligament strength and stability. When coupled with the absence of fibromodulin, tendon development is further impaired. Extracellular matrix niche is essential for tendon stem/progenitors [74]. It seems that dual loss of *Fmod* and *Bgn* also leads to dysfunctional stem/progenitor differentiation. It has been proposed that without these SLRPs, osteogenic growth factors are no longer sequestered away from tendon stem/progenitors, thus allowing for them to enter into ossification [71]. Continued utilization of this compound mutant *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ mouse should produce answers in regards to confirming this mechanism in the future.

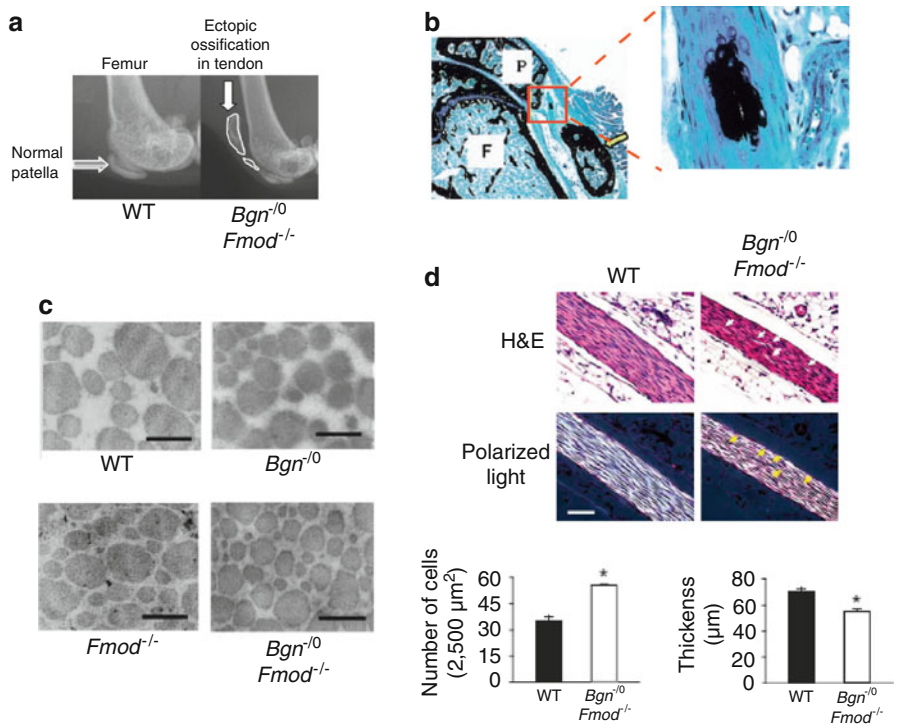

Fig. 13.4 Tendon structure is dramatically altered in *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ compound mutant mice. Tendons of the compound mutant mice demonstrate ectopic ossification. (**a**) Radiographs of 3-month-old mouse patellar tendons exhibit substantial ossification of the *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ quadriceps tendon. (**b**) Ectopic ossification can also be seen in Von Kossa-stained sagittal section of 6-month-old quadriceps tendons; the area of the image enclosed in the box is depicted alongside at a higher magnification. (**c**) Fibril diameters of *Bgn*$^{-/0}$, *Fmod*$^{-/-}$, and *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ quadriceps tendons are much smaller than those of wild type mice. (**d**) H&E staining of the patellar tendon demonstrate that the overall thickness of the *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ patellar tendons is small than that of wild type mice (P6). Moreover, under polarized light microscopy the collagen fibers within the *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ tendon were disorganized. Cell density in the *Bgn*$^{-/0}$ *Fmod*$^{-/-}$ is also much greater, further indicating that the absence of these SLRPs affects the extracellular matrix for cells within the tendon. Bars: (**c**) 200 nm, (**d**) 50 µm (Figure adapted with permission from: (**a**) the Scandinavian Journal of Medicine & Science In Sports (Kilts T. et al. Biglycan (Bgn) and fibromodulin (Fmod) in ectopic ossification of tendon induced by exercise and in rotarod performance. Scand J Med Sci Sports. 2009 August; 19: 536–546. © John Wiley & Sons, Inc., 71); (**b**, **c**) the FASEB Journal (Ameye L. et al. © Fedn of Am Societies for Experimental Bio, [64]); (**d**) Nature Medicine (Bi Y. et al. © Nature Publishing Group, [64, 71, 72])

Another compound mutant model used to study tendon development was generated after noticing expression changes in a single null mutation. Originally, when fibromodulin was targeted, a tendon phenotype was noted for the *Fmod*$^{-/-}$ mice [75]. Tail tendons of *Fmod*$^{-/-}$ mice contained fewer fiber bundles that are poorly organized and have an abnormal morphology [75]. Ultrastructural analysis of Achilles tendon from *Fmod*$^{-/-}$ mice showed collagen fibrils with irregular and rough outlines with thinner fibrils [75]. These results are suggestive of a role for *Fmod* in collagen fibrillogenesis and for compensation by *Lum* in *Fmod*$^{-/-}$ mice [75]. To discern the effect of *Fmod*$^{-/-}$ *Lum*$^{-/-}$ mutations on tendon development, a compound mutant mouse was generated [61]. Several distinct abnormalities were noted: (a) the premature presence of fibril diameter heterogeneity in the 4-day-old tendons; (b) an abnormally large number of small diameter fibrils present in the later stages; (c) irregular fibril profiles, indicative of abnormal lateral

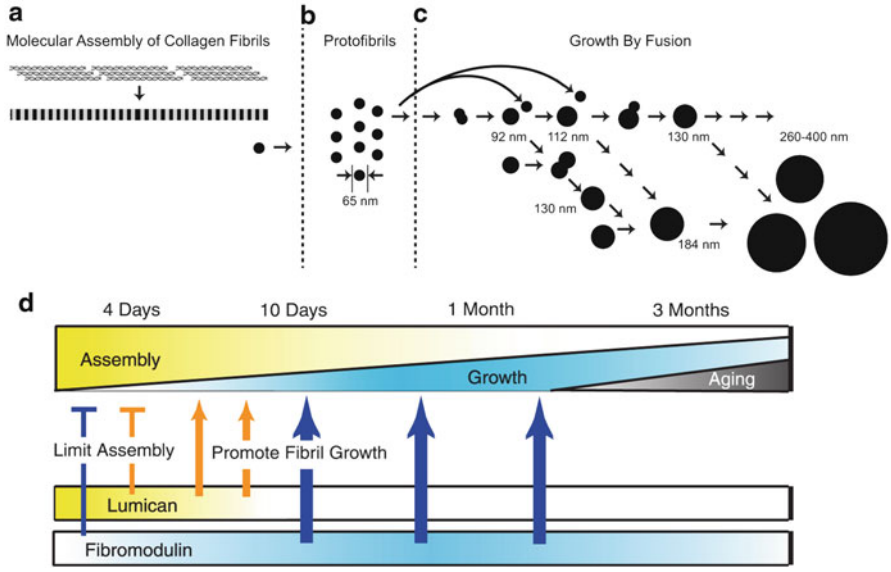

Fig. 13.5 Model for regulation of fibril growth by lumican and fibromodulin. The steps in collagen fibrillogenesis during tendon development are presented (**a**–**c**). (**a**) Early in fibril formation, the molecular assembly of collagen monomers into fibril intermediates occurs in the pericellular space; collagen molecules (*bars*) assembled into staggered arrays that form the fibril intermediates. At this stage, growth in length and diameter occur by accretion of collagen. (**b**) Through interactions with regulatory molecules like SLRPs and FACITs, the fibril intermediates are stabilized at a diameter of ~65 nm. (**c**) Fibril intermediates are the basic units in fibril growth; fusion of the fibril intermediates generates the mature fibril in a multi-step process. Progression through this growth process occurs both via additive fusion (*horizontal arrows*) and via like-fusion (*diagonally-oriented arrows*). (**d**) Assembly, growth, and aging can be considered as gradual processes. At early stages, assembly is the main event; however, its proportion gradually decreases to the minimum degree necessary for maintenance at maturation. Then the proportion of progressional growth increases gradually to ~1 month and decreases to maturation. The expression data for lumican and fibromodulin is illustrated as gradient *bars* at the *bottom* of panel (**d**). The phenotypes observed in mutant $Lum^{-/-}$, $Fmod^{-/-}$, and $Lum^{-/-}$ $Fmod^{-/-}$ mice indicate stage-specific regulatory mechanisms. At 4 days, both lumican and fibromodulin limit the assembly of the collagen monomers (*bars*). Characteristic of 10 days, progressional growth begins, and changes in both lumican and fibromodulin promote the transition from assembly to fibril growth by fusion (*thin arrows*). At later stages, only fibromodulin promotes the progressional growth steps (*thick arrows*) (This figure was taken from Ezura et al. © The Rockefeller University Press, [61])

association or a defect in molecular rearrangement after fusion; and (d) large numbers of very abnormal "cauliflower" fibrils at 1–3 months of age [61]. Thus, it appears that lumican and fibromodulin both influence initial assembly of protofibrils and the transition into fibril growth; moreover, fibromodulin, that is expressed in later stages, facilitates the progression through lateral growth, leading to mature fibrils (Fig. 13.5) [61]. The phenotypic changes to tendons in $Fmod^{-/-}$ and $Fmod^{-/-}$ $Lum^{-/-}$ mice lead to tendon weakness. Interestingly an absence of fibromodulin was required for the functional phenotype, but reduction in lumican from $Lum^{+/+}$ to $Lum^{+/-}$ to $Lum^{-/-}$ ameloriated the effect. As with compound mutant $Bgn^{-/0}$ $Fmod^{-/-}$ mice, there was joint laxity leading to osteoarthritis [63]. Small leucine-rich proteoglycans play an essential role in tendon development through regulation of lateral growth of collagen fibrils. Several mouse models have been developed to distinguish each SLRP's role in this process.

13.7 Beaded Filament-Forming Collagens

Collagen VI is ubiquitous within connective tissues, is found as an extensive filamentous network with collagen fibrils, and is often enriched

in pericellular regions. It assembles into several different tissue forms, including beaded microfibrils, hexagonal networks and broad banded structures [76–78]. Collagen VI has been proposed to integrate different components of the extracellular matrix, particularly allowing for an extracellular matrix-cell interaction [79]. In musculoskeletal tissue, collagen VI has proven to be essential; mutations have been shown to cause various forms of muscular dystrophy in humans [80]. *Col6a1*-null mice were generated and used to study the role of the gene in musculoskeletal tissue development [81–84]. Originally the *Col6a1*-null mouse was generated to study the pathophysiological mechanisms observed in human congenital myopathies such as those of Bethlem and Ullrich myopathies; [81] however, other phenotypes are also evident for tendon and bone and have recently been published [82–84].

Mice with a *Col6a1*-null mutation have muscle, bone, and tendon phenotypes. Without *Col6a1*, no collagen VI was produced in mutant mice [81–83]. Skeletal muscle of *Col6a1*-null mice exhibited necrosis and more pronounced muscle fiber diameter variation, particularly in the diaphragm, intercostal muscles, oblique and straight abdominal muscles, and femoral medial large muscles [81]. The *Col6a1*-null mouse myopathy manifested in reduced distances run by mice at least out to 1 year of age [81]. In the tendons of *Col6a1*-null mice, there are decreased fibril diameters and increased fibril density. Abnormal fibril structure is observed primarily in the pericellular region of mutant mice. This is associated with significant reductions in load and stiffness as well as increased matrix metalloproteinase activity [83]. In addition, fibroblast organization was severely disrupted in *Col6a1*$^{-/-}$ tendons; the fibroblasts and their processes were less attenuated and the fiber micro-domains were much smaller and less organized, thus leading to the development of smaller, less organized fibers [83]. These differences in the absence of collagen VI suggest that it is required for maintenance of cell shape, micro-domain structure and organization of fibrils into fibers during tendon development [83]. Likewise, bone of *Col6a1*-null mice was also severely affected [82, 84]. In these null mice, cancellous bone mass was reduced (by bone volume per tissue volume), and osteoblasts in the cancellous bone and cambium layer are misshapen, flattened instead of cuboidal with a disorganized border [84]. Moreover, cross-sections of tibial diaphyses from null mice demonstrated collagen arrangements predominantly parallel to longitudinal surface of the bone, while wild type mice showed collagen fibril alignment parallel in outer region and vertical in inner region [84]. An examination of *Col6a1*-null mouse tibial epiphysis showed that bone volume remained constant in null mice, though volume increased in the wild type [82]. As the epiphysis matured, cortical and trabecular bone densities remained relatively equivalent to immature wild type bone [82]. Moreover, subchondral bone thickness was decreased in the null mice [82]. Joint capsule thickness also was notably thicker in the *Col6a1*-null mice and visible ossification was occasionally noted; while overall articular degradation remained reduced in the *Col6a1*-null mice, more osteo- and chondrophytes were evident [82]. Clearly, from all of the phenotypic changes in the *Col6a1*-null mouse model, the importance of collagen VI to musculoskeletal structures has been confirmed.

13.8 Fibrillin

Fibrillins are glycoproteins that give rise to microfilaments. Fibrillins interact or associate with extracellular matrix proteins, microfibril-associated glycoproteins (MAGPs), elastins, and TGFβ-binding proteins [85]. Mutations in fibrillins cause Marfan syndrome in human patients [86, 87]. Thus, fibrillin-1 and −2 are subject to much investigation. Fibrillins have been found in skin, vasculature, and lung – all tissues containing elastin. In the canine flexor digitorum profundus, fibrillins were found with elastin in the tissue surrounding the outer layers of the tendon, as well as pericellularly around endotenon fibroblasts [88]. Fibrillin-1 was in the outer cell layers, while fibrillin-2 was found within the endotenon [88]. MAGP-1 and MAGP-2 were also found throughout the tendon but particularly at the insertion [88]. Likewise, fibrillins were found within canine cruciate ligaments [89].

Fibrillin-1 was found with greatest abundance in the epiligament; it was found pericellularly in the ligament in regions where elastin could be found, as well as throughout interfascicular regions [89]. Fibrillin-2 was found broadly throughout the ligaments, aligned parallel with collagen bundles; it was less commonly found with elastin within the ligament [89].

A null mouse for fibrillin-2 was generated; the tendons of this *Fbn2*$^{-/-}$ mouse contain less collagen cross-linking, though total collagen content and gross tissue morphology are unchanged relative to wild type tendons [90]. The *Fbn2*$^{-/-}$ mouse, however, did demonstrate fusion of third and fourth digits metacarpals and phalanges as well as shortening of tibiae, metatarsals, and phalanges [90]. Both phenotypic features of bone could be due to a lack of TGFβ-binding proteins associating with the extracellular matrix in the absence of fibrillin-2 [90]. Bone mass in 4-month-old *Fbn2*$^{-/-}$ femora was reduced relative to wild type femora, and there was an appreciative decrease in cancellous bone within vertebrae of 3- and 6-month-old *Fbn2*$^{-/-}$ mice [91]. Osteoblasts and mesenchymal stromal cells isolated from *Fbn2*$^{-/-}$ mice, even when supplemented with osteogenesis media, yielded only small numbers of bony modules in culture; accompanying expression profiles demonstrated decreases in bone marker expression, as *Osterix* (*Osx*) transcription factor expression was reduced [91]. Thus, *osterix-dependent* osteoblast maturation is impaired without *Fbn2,* that when absent makes TGFB proteins less bioavailable, leading to impaired bone maturation and mineralization [91]. Moreover, it should be noted that without *Fbn2* as a result of a mouse N-ethyl-N-nitrosourea (ENU) mutagenesis screen, limb strength was notably reduced due to muscle defects [92]. While it was very difficult to study *Fbn1*$^{-/-}$ mice because of neonatal death and aortic complications similar to what might be seen with Marfan syndrome, newly developed conditional models provide data that are suggestive of a role for *Fbn1* in osteoprogenitor differentiation [93]. Hence, while fibrillin mutations have yet to be implicated in tendon and ligament phenotypes or pathologies, because fibrillin-1 and −2 can be found in tendon and ligament, further studies might reveal their roles in these tissues.

13.9 Growth Factors

Growth factors play important roles in cell proliferation and matrix synthesis in tendon and ligament development and repair. The initial ectodermal cue in tendon development has been suggested to be a growth/differentiation factor such as GDF8, that has been demonstrated to induce Scleraxis (*Scx*) expression and tenocyte proliferation [94]. Moreover, in the first phase of tendon development, Fgf4 has been detected in myoblasts adjacent to those tendon primordia that have migrated [95]. In the chicken, the presence of Fgf4 maintains the expression of *Scx* and *tenascin-C* (*Tnc*) in tendon primordia [96]. Fgf8 has been detected in a proximo-distal spreading expression pattern throughout the second phase, after *Scx* expression is well-established and near the myotendinous junction within the tendon [97]. In the third phase, tendon development is promoted by Fgf4 from the muscle, that has been shown to affect *Scx* and *Tnc* expression [96]. Additionally, transforming growth factor-β3 (*TGFβ3*) is expressed in tendons until E15.5 when expression shifts to TGFβ1 that is notably a stage where regenerative capabilities of skin healing in the fetus diminish [98, 99]. Finally in the fourth phase (Stage 31 in chicken, E15.5-E19 in mouse), tendon insertions appear and *Scx* expression is seen throughout the tendons of the limb at a period when *TGFβ1* expression occurs [98, 100]. Clearly, many growth factors play a role in each phase of tendon development.

Like in development, growth factors affect tendon and ligament repair. TGFβ1 is expressed immediately after injury and remains at constant levels throughout repair, [101] though with acute tendon-to-bone injuries peak expression coincides with the period of greatest cell proliferation [102]. TGFβ1 is believed to act to stimulate cell migration, regulate proteinases, and stimulate collagen production, including the up-regulation of collagen III during the repair

process [101, 103]. Like TGFβ1, IGF1 and PDGF expression are thought to occur through much of the healing process to stimulate cell proliferation as well as matrix synthesis [101, 104]. FGF2 has been found within the bordering edges and within the lesion throughout the healing process with a peak at 7 days in rat and rabbit; its detection coincides with increases in tenocyte proliferation [101, 105]. Growth factors provide essential stimuli for tendon and ligament repair. In this chapter subsection, examples of mouse models useful for investigation of growth factors in tendon and ligament research are presented.

13.10 Transforming Growth Factor Beta

The importance of the transforming growth factor beta (Tgfβ) family in tendon development and repair is well-documented with studies focused on detection and quantification of expression of these growth factors. Gene targeted null mouse models have been created to study these growth factors. Mouse models described in this section include null mice for: *Tgfb1*, *Tgfb2*, *Tgfb3*, *Tgfbr2*, and *Tieg*. These models are important in analysis of the musculoskeletal impact of these genes on development.

Transforming growth factor beta genes have a significant impact on mouse development. *Transforming growth factor beta 1* (*Tgfb1*) has important roles in immune responses, inflammation, and healing [106]. An analysis of skin wounds from *Tgfb1*-null models revealed a delay in wound healing, including in inflammation, angiogenesis, and granulation [106]. Musculoskeletal studies of *Tgfb1* deficiency have not been described in the literature. The majority of $Tfgb2^{-/-}$ mice die at birth or shortly thereafter; mice were characterized to have heart (including valve), skeletal, corneal, inner ear, and urogenital defects [107]. Reduced biomechanical properties in $Tfgb2^{-/-}$ mice seem to be accounted for by defects in muscle and tendon; for example, limb extensor tendons were missing in the forelimb at E15.5 as well as tail tendons, though forelimb flexors were similar to wild type [108]. In $Tfgb3^{-/-}$ mice, tendons were not disrupted; however, $Tfgb2^{-/-}$ $Tfgb3^{-/+}$ mice demonstrated severe reductions in both forelimb flexors besides missing extensors [108]. When compound mutant $Tfgb2^{-/-}$ $Tfgb3^{-/-}$ mice were generated, embryonic lethality often occurred with midline fusion defects, blood vessel ruptures, several cardiac defects, missing intercostal and thoracic muscles [109, 110]. To determine the roles of *Tgfb2* and *Tgfb3* in tendon development, conditional *Tgfbr2* knockout embryos ($Tgfbr2^{Prx1Cre}$) were generated that essentially led to a musculoskeletal $Tfgb2^{-/-}$ $Tfgb3^{-/-}$ phenotype, since Tgfb2 and Tgfb3 act upon the receptor Tgfbr2 [108]. In these compound null mice, expression of *Scx*, *Tnmd*, and *Col1a1* was absent from the limb [108]. Tendons and ligaments throughout the $Tfgb2^{-/-}$ $Tfgb3^{-/-}$ compound mutant mouse and in the $Tgfbr2^{Prx1Cre}$ mouse were missing, particularly all limb tendons [108]. However, initial tendon progenitor proliferation was evident E10.5-E11.5 with tendon loss occurring at E12.5. Thus, while progenitor cell expansion occurs, differentiation is severely limited as tendon phenotypic maintenance is severely affected by the absence of TGFβ signaling [108]. Clearly tendon development is disrupted by the lack of *Tgfb2*, *Tgfb3*, and/or *Tgfbr2*. A TGFβ signaling-related mouse model was generated that was used to study the effect of TGFβ-inducible early gene (*Tieg*) inactivation, and thus Smad signaling, on flexor tendon repair [111]. Reactivity for TGFβ was delayed in the $Tieg^{-/-}$ mice after injury (3 days WT vs. 7 days $Tieg^{-/-}$), and levels of *Col1a1* expression were reduced throughout the repair process in $Tieg^{-/-}$ relative to wild type [111]. Future mouse models would greatly benefit from more sophisticated engineering of conditional and inducible mutations that could allow for the study of tendons and ligaments post-embryonic development.

13.11 Growth/Differentiation Factors

Growth/differentiation factors (GDFs) are a subclass of bone morphogenetic proteins (BMPs) that are related to transforming growth factors (TGFs). Several GDFs have been implicated in

musculoskeletal development, particularly in tendon development; these include *Gdf5*, *Gdf6*, *Gdf7*, and *Gdf8*.

The first GDF mutation examined was *Gdf5*. The *Gdf5*-null mouse was discovered as a natural mutation in 1952 in what was at that time designated a *brachypodism* mouse model; thus, the mouse is often called the *bp* mouse [112]. The affected gene was determined to be GDF5 some 42 years later [113]. These mutant mice possess many musculoskeletal phenotypic features: smaller paws, shorter long bones with greater effect on hindlimbs, and skeletal defects more notable with distal elements [112]. It was concluded that, from the nature of the defects, most likely the mutation affected genes involved in embryonic limb mesenchymal condensation [112, 114]. Mutations of *Gdf5*-homologue *cartilage-derived morphogenetic protein-1* (*CDMP1*) share phenotypic features, and this mutation was described clinically as Hunter-Thompson or Grebe type chondrodysplasias and as brachydactyly C [112]. GDF5 has been well-studied in chondrogenesis; it is important to the mesenchymal condensation process in embryonic development, and it has an effect on chondrocyte proliferation in early chondrogenic cartilage structures [112]. An examination of *bp* mouse femora indicated that medullary area, cortical area, polar and bending moments of inertia, and torsional constant were all lower than for a wild type mouse, even after normalizing for size differences since the *bp* mice are smaller [115]. While bones of *bp* mutants demonstrated lower effective shear modulus, tissue composition was little affected [115]. Thus, *bp* mutant bones were smaller, structurally weaker, and less stiff than wild type bones. Similar mutant mice had disorganized collagen fibril alignment, though total collagen composition was similar to wild type [116]. Collagen composition was affected, though, in Achilles tendon of *bp* mutants [117]. More specifically, mature *bp* mouse Achilles tendons contained 40 % less collagen compared to control (heterozygote) mice [117]. In addition, Achilles tendons of *bp* mice had a smaller maximum load to failure, among many other mechanical testing parameters, that when combined suggest that biomechanically the tendons in *GDF5*-null mice are compromised [117]. Furthermore, *bp* mature mutant tendons demonstrated ultrastructural changes in collagen fibrils, including more heterogeneity in fibril size and a shift to slightly smaller fibrils [117]. When the Achilles tendons of mature *bp* mutant mice are injured by tenotomy and then repaired with suture, healing was delayed; cellular proliferation, collagen and proteogylcan syntheses were all delayed [118]. Moreover, revascularization and collagen organization were delayed with more fatty tissue initially found in the tenotomy site of the *bp* mutant, and biomechanically the injured mutant tendon was weaker until about 12 weeks post-tenotomy [118]. In seven decades of research on the *bp*/*Gdf5*-null mutant mouse, there has been a great deal of evidence to show that *Gdf5* is essential to most musculoskeletal tissues.

Several studies have been performed to define the roles of *Gdf6*, *Gdf7*, and *Gdf8* in musculoskeletal development, particularly in regards to tendon biology, using gene targeted null mouse models. Tail tendons of *Gdf6*-null mice have about 30 % less collagen than wild type mice; this compositional difference was associated with less stiff tendons with qualitatively smaller fascicles that appeared to be crimped at greater angles [119]. Tail tendons of *Gdf6*-null mice were less stiff with smaller fascicles [119]. Effects of the *Gdf6* mutation in the null mice also appear to be somewhat sexually dimorphic, with more noticeable tail tendon phenotypic differences in males [120]. Overall, it appears that *Gdf6* affects collagen content and organization in developing tendons. Unlike *Gdf6*, it appears that absence of *Gdf7* does not affect collagen content in tendons [121, 122]. Moreover, *Gdf7*-null mice demonstrate no difference in small leucine-rich proteoglycan (SLRP) content, though collagen fibril diameters were slightly smaller in the null tendons [121]. In tail tendons, *Gdf7*-null mice demonstrated increased GDF5 protein levels, that could be compensating for the missing GDF7 [122]. Finally, the roles of *Gdf8*, or myostatin (MSTN), in muscle biology have been well studied. The effects of the loss of

Gdf8 in null mice, known as *MSTN*$^{-/-}$, were recently described; studies have shed light on the role of *Gdf8* in tendon development [94]. Tendons from *MSTN*$^{-/-}$ mice were smaller than wild type mice, and they were biomechanically much stiffer, and more brittle [94]. An examination of tendon fibroblasts indicated that, as with muscle, these cells express myostatin receptors that are capable of activation (MAPK and Smad signaling) upon myostatin treatment [94]. Activation of myostatin receptors induces tendon fibroblast proliferation; without *Gdf8*, fibroblast density decreases in the *MSTN*$^{-/-}$ tendons [94]. Moreover, activation of MAPK signaling by myostatin affected fibroblast tendon expression of scleraxis, tenomodulin, and collagen I [94]. These findings suggest that myostatin, possibly produced from closely positioned muscles, may play a role in initiating and/or maintaining scleraxis expression in the earliest stages of tendon development. Tendons and ligaments are greatly affected by the family of growth/differentiation factors. These mutant mouse models have been helpful so far in allowing for better characterization of tendon development. Certain changes in the mouse models in the future could better describe the roles of this family of growth factors in all phases of tendon biology, particularly during repair.

13.12 Transcription Factors

Transcription factors provide a critical regulatory step in determining the fate of musculoskeletal primordial cells and their differentiation and development into tendons and ligaments. They regulate the expression of genes that have a direct impact on the development and phenotype of a connective tissue. Two transcription factors important in tendon and ligament development are *Scleraxis* (*Scx*) and *Mohawk homeobox* (*Mkx*) gene. The tenogenic development and maintenance roles of *Scleraxis* and *Mohawk* are featured in Fig. 13.6. Recently, a pair of transcription factors *Early growth response 1* and *2* (*Egr1* and *Egr2*) have been found to be important for tendon development.

13.13 Scleraxis

Scleraxis (*Scx*) is a basic helix-loop-helix transcription factor expressed first in the developing embryo. *Scleraxis* is expressed in the somite sclerotome region in regions accounting for both the axial and appendicular portions of the skeleton, including the limb buds [100, 123, 124]. Early in embryonic development expression of *Scx* and *Sox9* is co-localized, but soon thereafter expression of the two genes delineates tendon from cartilage [123, 125]. Expression of *Scx* is thereafter associated with tendons and ligaments [123] and is further stimulated by Ets transcription factors *Pea3* and *Erm* [126]. Scleraxis has been implicated in the up-regulation of *Tenomodulin* (*Tnmd*) expression as a late marker in embryonic tendon development; [127] of *Tnmd* and *Col1a1* with patellar tendon repair; [128] and of *Tnmd*, *Col1a1*, *Dcn*, and *Fmod* expression in MSCs with lentiviral transgenic *Scx* expression to convert the cells into tendon progenitors [129]. Thus, *Scx* expression has been demonstrated in tendons and ligaments in development, repair, and in vitro all when cells are differentiating in tendon or tendon-like cells. Two types of models were generated to further elucidate Scx function; transgenic reporter models and null mouse models.

Transgenic reporter mouse lines permit the analysis of temporal and spatial expression of *Scleraxis*. Expression of an easily detectable reporter is driven by the *Scx*-promoter region. Three *Scleraxis* transgenic model mice have been created: *Scx/lacZ*, *ScxAP*, and *ScxGFP* [125, 130]. Reporter expression in these transgenic models recapitulates normal *Scx* expression in the mouse (Fig. 13.7). Several studies have been completed that utilize *ScxGFP* transgenic mice [108, 131, 132]. This is particularly useful when combined with other models. For example, tendon phenotypes were followed for *Tgfb2*$^{-/-}$ mice using the *ScxGFP* transgene; missing extensor tendons were well visualized by the lack of tendons of the forelimb in the *Tgfb2*$^{-/-}$ mice [108]. *Tgfb2*$^{-/-}$ *Tgfb3*$^{-/+}$ embryos were missing extensor tendons and had severely reduced *ScxGFP*

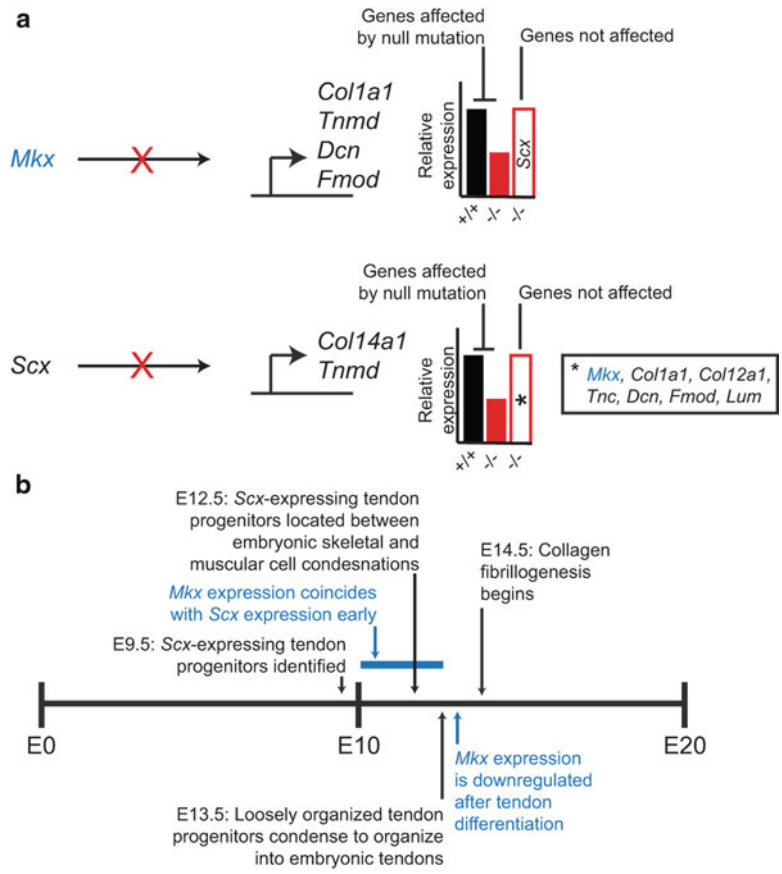

Fig. 13.6 Roles of Scleraxis and Mohawk in tendon differentiation. (**a**) Scleraxis (Scx) and Mohawk (Mkx) are essential for tendon development. Studies of *Mkx*$^{-/-}$ and wild type mice demonstrated that expression of *Col1a1* and *Tnmd*, *Dcn*, and *Fmod* are regulated by *Mkx*; however, *Mkx* does not seem to have a role in *Scx* expression as *Scx* transcript level do not change in *Mkx*$^{-/-}$ mutants. Studies of Scx$^{-/-}$ and wild type mice demonstrated that *Scx* affected tendon development and formation overall, as well as expression of *Col14a1* and *Tnmd* specifically. In *Scx*$^{-/-}$ mutants, expression of *Mkx*, *Col1a1*, *Col12a1*, *Tnc*, *Dcn*, *Fmod*, and *Lum* seem to be unaffected. (**b**) A timeline of tendon development shows that *Mkx* expression occurs early in tendon development and is downregulated after tendon differentiation, while *Scx* expression persists from tendon progenitor origination into and through development

expression [108]. For compound mutant *Tgfb2*$^{-/-}$ *Tgfb3*$^{-/-}$ mice, no tendons were detected at any level other than digits [108]. The effect of absence of *Scx* on tendons in the middle ear was examined by breeding *Scx*$^{-/-}$ mice with *ScxGFP* reporter mice as cells with a tendon lineage could be followed to define changes in middle ear tendon differentiation in *Scx*-null mice [132]. The *ScxGFP* mice also were used to track expression of *Scx* ahead of migration of fibroblasts from the epitenon into the superficial regions of tendon fascicles with exercise and corresponding expression of tenomodulin and collagen I [131]. Thus, the transgenic *ScxGFP* mouse revealed a new role of *Scleraxis* in post-exercise adaptation of tendons [131]. Clearly, these transgenic models offer the ability to track the lineage of tendon progenitor cells, to follow tendon formation, and to investigate the functional roles of genes associated with tendon development, maturation, and repair.

The availability of *Scleraxis*-null mice allows for analysis of the specific roles of *Scx* in all aspects of tendon and ligament biology and pathobiology. Viable *Scleraxis*-null (*Scx*$^{-/-}$) mice were first reported by Murchison et al. (Fig. 13.7) [133]. Within this model, severe tendon disruption

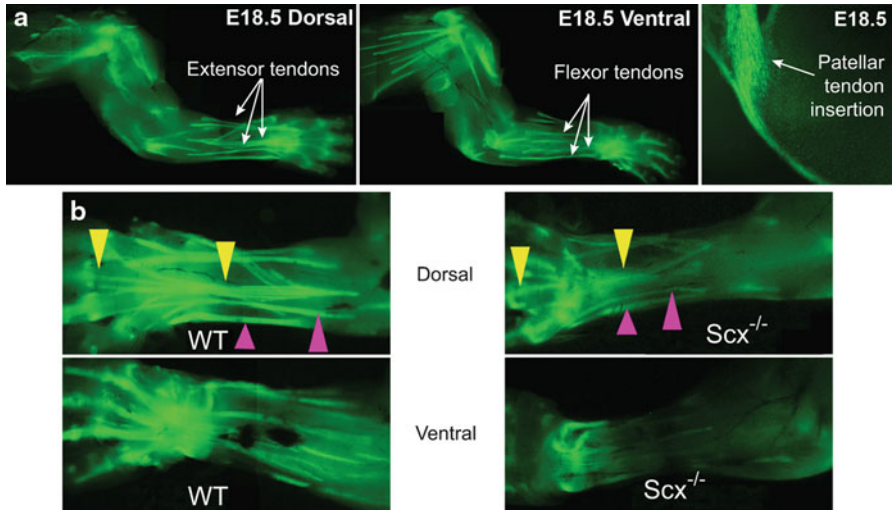

Fig. 13.7 The ScxGFP transgenic mouse model. (**a**) The generation of a transgenic mouse in which a green fluorescent protein (GFP) gene is fused with the Scleraxis (Scx) promoter allows for tracking tendon development via GFP-producing tenocytes. (**b**) In order to follow tendon development in *Scx*$^{-/-}$ mutant mice, the ScxGFP mouse was utilized; this resulted in strong GFP expression in tendon and ligaments and allowed for the discernment of *Scx* regulation of tendon formation. Note that relative to wild type mice, proximal forearm tendons did not exist in the *Scx*$^{-/-}$ mice while tendons of the paw were still detected via ScxGFP (This figure was adapted with permission from Pryce et al. © John Wiley & Sons, Inc., [130] and Murchison et al. © The Company of Biologists, Ltd. [133])

was present at all levels, particularly for force-transmitting and intermuscular tendons. Grossly, the paw or autopod was locked in dorsal flexure, leaving the mice to walk on their wrists and unable to grip. Back muscles were severely impaired, and the tail was immobile [133]. Many limb tendons were completely missing, and those tendons that were present often were shortened [133]. The tail tendon was missing, yet the ligamentous annulus fibrosus between each vertebra was not affected [133]. Scx$^{-/-}$ mice were crossed with *ScxGFP* transgenic mice to gain a better understanding of phenotypic findings. For example, intermuscular tendons – tissue that interconnects two different muscles – were missing; however, the broad tendon of the diaphragm was present, though none of the tendon cells were *ScxGFP*-positive [133]. Thus, in some cases functional connective tissue still formed. In the case of the diaphragm, expression of tenascin-C, collagen XII, and collagen I were maintained, though collagen I content was reduced [133]. Moreover, short-range anchoring tendons and ligaments were not affected in Scx$^{-/-}$ mice. Intercostal tendons and cruciate knee ligaments were intact with normal morphology and expression of collagen I [133]. While the patellar ligaments, that connect the patella and the tibia, were intact, the patellar tendon, was completely missing [133]. Additionally, while the tail tendon was absent, the ligamentous annulus fibrosus was intact. The *Scx*$^{-/-}$ mouse model demonstrates the important regulatory roles of *Scx* in tendon progenitor biology as well as development, maturation and pathophysiology.

13.14 Mohawk

Mohawk (*Mkx*) has a regulatory role in tendon differentiation [134]. *Mohawk* (*Mkx*) is a member of a superclass of atypical homeobox genes known as the "three amino acid loop extension" superclass. *Mkx* is expressed in developing tendons. Determinations of its specific role in tendon biology were investigated in *Mkx*$^{-/-}$ mice [134, 135]. Tendons of *Mkx*$^{-/-}$ mice were smaller compared to wild type controls, though cruciate

ligaments were unchanged [134, 135]. Tendon crimp was missing in tail tendons of *Mkx*$^{-/-}$ mice [135]. Typical tendon fibril diameter growth for forelimb flexor digitorium profundus was disrupted in *Mkx*$^{-/-}$ mice, thus resulting in smaller fibril diameters when compared to wild type tendons [135]. These phenotypic changes affected the tensile strength of *Mkx*$^{-/-}$ Achilles tendons because of reduced tendon mass [134]. Moreover, tendon sheaths were thicker and more cellular in *Mkx*$^{-/-}$ mice [135]. Expression of *Scx* was unaffected by loss of *Mkx* and vice versa; thus, these genes seem to function independently [135]. The loss of *Mkx* was associated with reduced expression of *Col1a1*, *Col1a2*, *Dcn*, *Fmod*, and *Tnmd* [135]. Thus, transcription factor *Mkx*, as well as its effects on tendon and ligament development and collagen organization, all have been defined using a *Mkx*$^{-/-}$ mouse model.

13.15 Transcription Factors Early Growth Response 1 and 2

Early growth response-like transcription factor *Stripe* was first characterized to regulate tendon primordial cells in Drosophila [136, 137]. There are several members in the vertebrate family of early growth response (*Egr*) transcription factor genes. Typically *Egr1* and *Egr2* expression is seen to peak when tendon progenitors are active and differentiating [138]. *Egr1* and *Egr2* expression can be stimulated when tissue is supplemented with Fgf4 [138]. *Egr1*$^{-/-}$ and *Egr2*$^{-/-}$ mice were used to discern the role of these genes on tendon development [138]. In the absence of *Egr1*, levels of *Tnmd* and *Col14a1* decrease significantly, while levels of *Col1a1* and *Col1a2* are significantly reduced in the absence of *Egr2* [138]. In the absence of either *Egr1* or *Egr2*, numbers of fibers (collagen fibril bundles) in embryonic tendons were reduced by 30–40 % [138]. Compound mutant *Egr1*$^{-/-}$ *Egr2*$^{-/-}$ mice did not have completely abolished *Col1a1* expression; thus, other factors like *Scx* and *Mkx* play important roles in collagen I synthesis [138]. Still, mouse models for *Egr1* and *Egr2* demonstrate the unique role of early growth response genes in tendon development and a useful tool for further investigation.

13.16 Cell-Membrane Proteins Tenomodulin and CD44

Cell-membrane proteins tenomodulin and Cd44 play important functional roles in tendons. They are often used as phenotypic markers for tendons. Recent development of null mouse models has provided tools for further investigation of these proteins that are necessary to define their function.

13.17 Tenomodulin

Tenomodulin (*Tnmd*) is expressed in many avascular or hypovascular musculoskeletal tissues in cells of mesenchymal origin, such as tendons and ligaments [139]. Expression of *Tnmd* is regulated by *Scleraxis* [127]. Retroviral expression of *Scx* in cultured chicken leg tenocytes resulted in upregulation of *Tnmd*; however, expression was not upregulated in cultured chicken tibiotarsal chondrocytes [127]. A *Tnmd*$^{-/-}$ mouse was developed by targeted recombination [140].. The *Tnmd*$^{-/-}$ mouse served as an excellent model for the examination of the role of *Tnmd* in tendon maturation. Tendons of *Tnmd*$^{-/-}$ mice showed decreases in cell proliferation within the embryo with a similar trend up to P14, at that time point levels of collagen I and decorin were both comparable to wild type tendons [140]. Levels of collagen III were consistently weaker in newborn, 1-week-old, and 2-week-old mice for *Tnmd*$^{-/-}$ mice [140]. Achilles tendons of 6-month-old *Tnmd*$^{-/-}$ mice had a uniform distribution of collagen fibril diameters; however, there was a greater range in the variation of diameters and cross-sections of fibrils appear uneven and rough relative to the round and smooth fibrils of wild type mice [140]. These findings implicate *Tnmd* in affecting tendon cell proliferation during embryonic development and subsequent postnatal tendon maturation; thus, the *Tnmd*$^{-/-}$ mouse

model is valuable for studying several aspects of tendon biology and pathobiology.

13.18 Cd44

Cd44 is a polymorphic type I transmembrane glycoprotein that is involved in cell-cell and cell-matrix interactions, leukocyte homing and activation with inflammation, cell migration, and extracellular matrix assembly [141]. Its ligand is hyaluronic acid (HA). Cd44 has been shown to bind to collagen I and VI [141, 142]. When added to rabbit flexor tendon injuries, HA demonstrated a dose-dependent inhibition of cell proliferation for tendon fibroblasts, yet HA stimulated greater migration of the tendon fibroblasts [143]. These findings suggest an important role for the Cd44-HA interaction in cells of the tendon and ligament. A Cd44-null mouse was used to study the effects of this interaction [144]. Biomechanics of patellar tendons of mature Cd44-null mice were unchanged compared to wild type [144]. Also, no expression differences were seen for *Col1a1* and *Col3a1*, *Dcn*, *Bgn*, *Tgfb1*, *Tgfb3*, *bFgf*, *Pdgfb*, and *Has2* [144]. After injuring patellar tendons, tendon healing was improved in the null mice when comparing material properties as well as expression of genes conducive to better regenerative healing [144]. Specifically, expression of *Tgfb3*, *bFgf*, and *Dcn* were increased in the first 7 days postinjury in the Cd44-null tendons compared to wild type [144]. Expression of *Col3a1, Bgn*, *Tgfb1*, and *Pdgfb* had greater increases early post injury, and *Has2* increases were evident after 3 days, though no appreciable differences were seen for *Col1a2* and *Il1β* [144]. Material properties and expression data from this null model suggest that participation of Cd44 in healing does more to impede the healing process for the patellar tendon. Information gained from the null model might indicate that cell proliferation is more valuable than cell migration in full-thickness partial patellar tendon transections. Further studies with this model are required to determine why the improved mechanism occurs in the absence of Cd44.

13.19 The Future of Mouse Models

Most of the mouse models described within this chapter are null for specific genes. Null mouse models are extremely useful in understanding the role of a gene, particularly within development. However, as a null mutant mouse matures, the cumulative effects of the absence of a gene could make it difficult to completely understand its role in a specific situation, e.g. healing and conditioning with exercise. Moreover, sometimes null mutations are embryonic lethal or contribute to early postnatal death, thus making analysis of mature and aged tissues impossible. While in the past heterozygote null mutants were used to remedy these issues, today many new mouse models are being developed that are considered conditional and/or inducible mutant models. Furthermore, in order to follow cell fate or activation of expression for specific genes, transgenic mouse models have been developed that target gene promoter/reporter fusions. These two types of mouse models are proving to be useful resources; their utilization in tendon and ligament biology will provide additional tools necessary to address many key unanswered questions about musculoskeletal diseases and injuries.

13.20 Transgenic Mice

Transgenic mice are being developed for stage- and tissue-selective expression of a target gene or reporter gene. By utilizing this strategy, over expression of a target gene to determine the effect of its expression by developmental stage and/or by tissue, or they are able to induce the expression of a reporter such that the promoter's regulatory roles may be investigated. One such example that is relevant to tendon and ligament biology would be the *ScxGFP* mouse [130]. This line, along with *ScxAP* and *Scx-lacZ* allow for tendon and ligament progenitor cells to be followed through to maturity [125, 130]. Moreover, in the presence or absence or other genes, the transgenic reporters allow for the discernment of cell

status within mutant models, particularly in regards to development and differentiation [133, 134]. Cells from these mice are also easily accessible for isolation and in vitro manipulation. Thus, when these mice are used in combination with null, conditional, or inducible mutants, an improved understanding of that gene might be visualized and interpreted with the understanding of the typical spatiotemporal expression of that promoter-driven reporter. The effects of mutated forms of genes might also be examined. Transgenic mice also are generated that expresses a truncated or otherwise mutated form of a protein. Thus the role of a region of that protein might be investigated in combination with the native protein as in wild type, without the native protein as in a targeted null mutant, or to see if there is an interaction with another targeted gene null mutant [56, 145]. These models will shed light on dominant-negative interactions between mutated and wild type proteins often important in genetic diseases. However, when transgenic mice are generated, there are limitations to the control of how many copies of transgenes get incorporated. This could lead to dose dependence or copy number variation amongst generated mouse lines. Equally as concerning is the site of the insertion of the transgene; *cis* and *trans* genomic regulatory elements near the transgene could affect its expression. Bacterial Artificial Chromosome (BAC) transgenesis has been utilized in an effort to overcome these limitations. The large size of BACs reduces the likelihood of incorporation of multiple copies and increases the chances that expression patterns will be more similar to that of the endogenous gene [146]. Transgenic mouse models add another level of sophistication to the questions that might be asked by tendon and ligament biologists.

13.21 Conditional and Inducible Mice

Other mouse model strategies are conditional and inducible knockout and/or knock-in approaches. Because targeted null mutants are sometimes embryonic lethal or the cumulative changes associated with the target gene deletion affect the understanding of that gene's role in specific responses, e.g., healing or conditioning, researchers are finding it increasingly more useful to not only target the gene, but also when and where it is expressed.

Conditional and inducible gene targeting for mutation relies upon four steps that lead to specified control of the expression of a gene (Fig. 13.8). Typically, mutation leads to gene inactivation or the absence of the protein encoded by the gene. Targeting of the gene relies upon recombination of a targeting vector in the genome of the mouse. Within this targeting vector, there are four components: (a) the mutated target gene, (b) a positive selection gene to allow for selection of intermediate mouse stages post-recombination, (c) recombinase recognition sites (e.g., *LoxP* and *FRT* sites) flanking the positive selection gene as well as the site to be mutated within the gene, and (d) upstream 5′ and downstream 3′ flanking regions of the targeted gene that are homologous arms that will initiate homologous recombination. After successful homologous recombination of the targeting vector within an embryonic stem (ES) cell and removal of the positive selection gene, the intermediate recombinant mouse is bred with a transgenic mouse containing a promoter/recombinase fusion such that the recombinase (e.g., *Cre* recombinase) will be activated in a tissue-, stage-, or inducer specific manner, depending upon the promoter utilized. Whenever or wherever the recombinase is expressed, the targeted site will be removed, leaving the recombinase recognition site and thus completing the mutation to the targeted gene. Conditional mutant model strategies have been used to examine the effect of *Tgfbr2* in developing limb bud mesenchyme [147, 148]. Future studies into tendon and ligament development could rely upon *Scx*-, *Sox9*-, *Prx1*-, or *Col1a1-Cre* recombinase-driven conditional mutations, which have already been generated [147, 149–151]. Moreover, common inducible gene targeting strategies include Cre recombinase-Estrogen Receptor, or Cre-ERT, [152] and tetracycline-controlled transactivator, or tet-ON/tet-OFF [153]. Future studies into ligament and tendon biology could allow for gene targeting or cell tracking during develop-

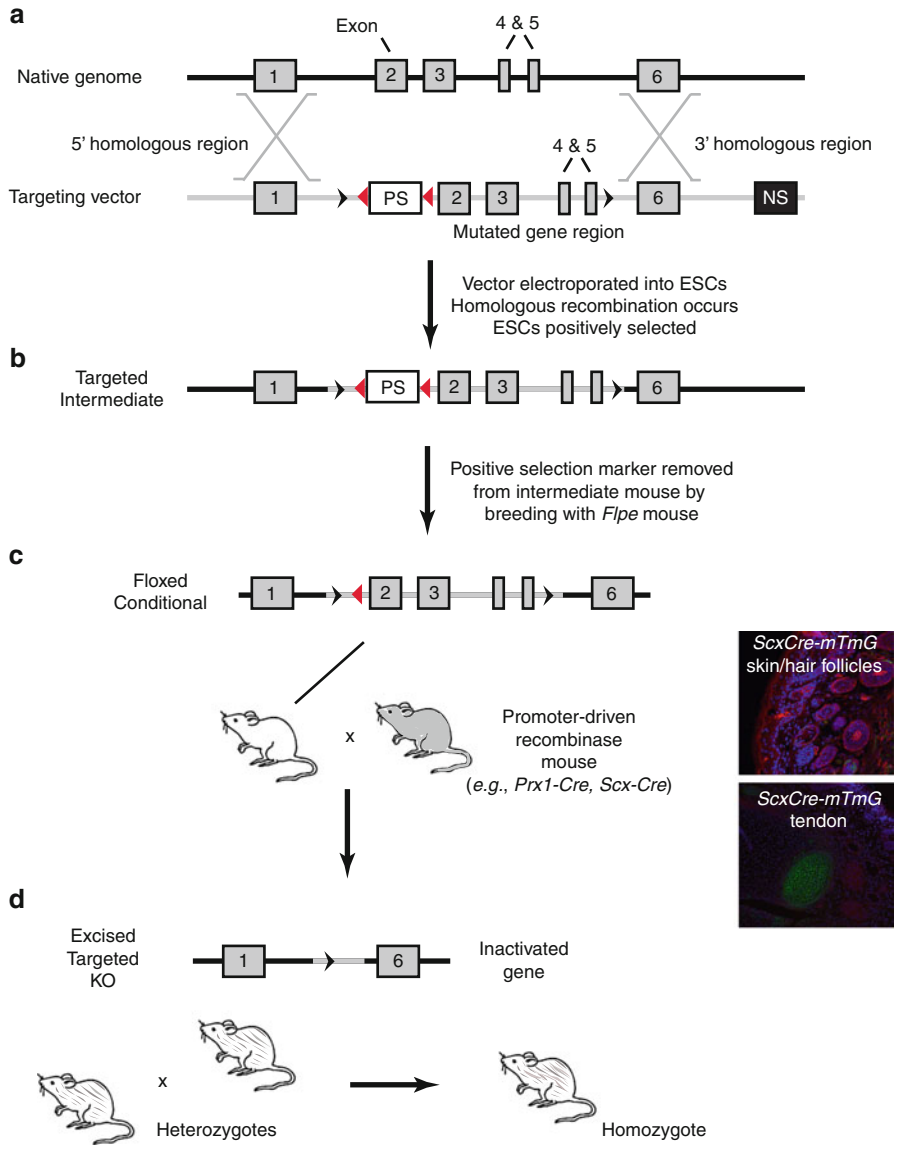

Fig. 13.8 Generation of a mouse with conditionally targeted gene. (**a**) To generate a conditionally targeted gene mutant mouse, a targeting vector is designed that contains 5′ and 3′ homologous regions that will allow for homologous recombination of the vector with the native genome. Also within this vector are a positive selection gene (PS) flanked by *FRT* sites (*red arrowheads*), for example, to allow for selection of intermediate mouse stages post-recombination, a mutated region of the target gene flanked by LoxP sites (*black arrowheads*), and a negative selection gene (NS) which should not be detectable in recombinants. The vector is electroporated into embryonic stem cells. Recombinant stem cells are injected into mouse blastocysts. (**b**) The mice produced need to have the positive selection cassette removed which can be done in the animal or ES cells using a distinct recombinase site such as *FRT*.(**c**) The floxed conditional mouse generated has a fully functional allele with a targeted site flanked by *LoxP* elements. (**d**) When the floxed conditional mouse is bred with a transgenic mouse containing a specific promoter-driven recombinase (*Scx-Cre/mTmG* shown to demonstrate specificity for Scx-Cre in the tendon and not in the skin for a transgenic breeding line), the resulting progeny are excised target knockout (KO) mice and will contain the targeted gene mutation (heterozygote) and the promoter-driven recombinase. Follow-up breeding of heterozygote excised target KO mice will lead to homozygote excised target KO progeny

ment, conditioning, and injury. Sophisticated improvements in transgenic and mutant models will allow for more specific questions to be answered about tendon and ligament biology.

13.22 Summary

Mutant mouse models have been valuable resources for gaining an improved understanding of tendon and ligament biology. Phenotypes for some mutant mouse models mimic clinical manifestations of heritable disorders like Ehlers-Danlos Syndrome, Osteogenesis Imperfecta, Marfan Syndrome, and chondrodysplasias. Mutant mouse models also are used to distinguish the roles of genes in procollagen synthesis, collagen processing or organization, as well as the downstream effects on the rest of the musculoskeletal system when deficiencies lead to structural and functional weaknesses in tendons and ligaments. Mouse models for fibril-forming collagens are used to elucidate the roles of these collagens are to the structure and function of musculoskeletal tissues. Mouse models for regulatory molecules like FACITs and SLRPs are valuable tools in studies analyzing the mechanisms driving linear and lateral fibril growth as well as higher order assembly during tendon growth and maturation. In addition, growth factor mouse models are useful for defining the specific regulatory processes involving one or more growth factors in cell proliferation and matrix synthesis in tendon and ligament development and repair. Likewise, mouse models for transcription factors are essential to reveal just how much the fate of musculoskeletal primordial cells relies upon these factors for their differentiation and development into tendons and ligaments. Additionally, mouse models for the membrane-bound proteins tenomodulin and Cd44 demonstrate their important role in cell-cell and cell-matrix interaction. Finally, conditional and inducible mouse models allow for specific questions to be answered about tendon and ligament biology as regulation of expression of genes can be spatio-temporally controlled. Mouse models offer nearly limitless potential for researchers seeking to better understand tendon and ligament biology.

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Connective Tissue Disorders in Domestic Animals

14

Jaroslava Halper

Abstract

Though soft tissue disorders have been recognized and described to some detail in several types of domestic animals and small mammals for some years, not much progress has been made in our understanding of the biochemical basis and pathogenesis of these diseases in animals. Ehlers-Danlos syndrome described in dogs already in 1943 and later in cats affects mainly skin in these animals. The involved skin is thin and hyperextensible with easily inflicted injuries resulting in hemorrhagic wounds and atrophic scars. Joint laxity and dislocation common in people are less frequently found in dogs. No systemic complications, such as organ rupture or cardiovascular problems which have devastating consequences in people have been described in cats and dogs. The diagnosis is based on clinical presentation and on light or electron microscopic features of disorganized and fragmented collagen fibrils. Several cases of bovine and ovine dermatosparaxis analogous to human Ehlers-Danlos syndrome type VIIC were found to be caused by mutations in the procollagen I N-proteinase (pnPI) or *ADAMTS2* gene, though mutations in other sites are likely responsible for other types of dermatosparaxis. Cattle suffering from a form of Marfan syndrome were described to have aortic dilatation and aneurysm together with ocular abnormalities and skeletal involvement. As in people mutations at different sites of bovine *FBN1* may be responsible for Marfan phenotype. Hereditary equine regional dermal asthenia (HERDA), or hyperelastosis cutis, has been recognized in several horse breeds as affecting primarily skin, and, occasionally, tendons. A mutation in cyclophilin B, a chaperon involved in proper folding of collagens, has been identified in some cases.

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Degenerative suspensory ligament desmitis (DSLD) affects primarily tendons and ligaments of certain horse breeds. New data from our laboratory showed excessive accumulation of proteoglycans in organs with high content of connective tissues. We have identified an abnormal form of decorin with altered biological activity in these proteoglycan deposits, and more recently changes in processing of aggrecan were found by us and other investigators.

The naturally occurring diseases of soft tissues in domestic animals described here have a potential to serve as good models for analogous human diseases. This is the case particularly relevant to dogs as a half out of the more than 400 naturally occurring hereditary canine diseases has the potential to serve as a model for human disease.

Keywords

Ehlers-Danlos • Dogs • Cats • Dermatosparaxis • Cattle • Sheep • HERDA • Hyperelastosis cutis • Horses • Degenerative suspensory ligament desmitis (DSLD)

Soft tissue disorders have been recognized and described to some extent in several types of domestic animals and small mammals such as rabbit [1, 2]. Whether the relative paucity of cases in the literature is due to naturally low incidence of this group of diseases in domestic animals, or whether there is little incentive for studies in these animals is not clear. Though Ehlers-Danlos syndrome was first described in dogs as cutaneous asthenia already in 1947 [3], and though it is recognized that dogs are very suitable to serve as models for numerous human hereditary diseases and cancer [4] not many comprehensive studies have been done on connective tissue diseases in this species. Ehlers-Danlos affects preferentially certain breeds such as dachshunds, boxers, German shepherds and St. Bernards [5, 6] with a dominant pattern of inheritance [7]. Unlike in people, skin carries the brunt of the disease in dogs. It is thin and hyperextensible with easily inflicted injuries resulting in hemorrhagic wounds and atrophic scars [5, 8, 9]. Skin folds in the extremities, downy "chicken-like" skin, and "cigarette paper" scars are features found in dogs as well as in people suffering from Ehlers-Danlos syndrome, whereas joint laxity and dislocation common in people are less frequently found in dogs [5, 8]. Organ rupture and/or dissecting aneurysm, two dramatic and catastrophic events in people, have not been identified in dogs and other species [8]. Microscopic examination of the affected canine dermis usually shows fragmented and disorganized collagen fibers and fibrils and low number of poorly organized elastic fibers [5, 8].

Cats are occasionally affected by cutaneous asthenia or Ehlers-Danlos as well [10]. As in dogs their skin is hyperextensible and fragile because of decreased tensile strength. Dominant pattern of inheritance has been documented in some, but not all afflicted cats [10–13]. Electron microscopic examination reveals disorganized packing of collagen fibrils into fibers in the dermis, and the presence of fibrils with abnormally large or small diameters [10]. The diagnosis of cutaneous asthenia or Ehlers-Danlos in dogs and cats is based on clinical presentation and electron microscopic features of collagen fibrils rather than on the detection of a specific, as of yet to be identified, underlying biochemical defect.

The limited extent of the disease in cat makes it more similar to so called dermatosparaxis described in cattle [14] and sheep though the mode of inheritance of the latter has been determined to be autosomal recessive [15, 16].

Dermatosparaxis has been characterized to more detail in White Dorper and Dorper sheep where it has been recognized to be analogous to type VII C Ehlers-Danlos syndrome [17, 18]. The disorder is limited to skin which is quite fragile due to subcutaneous accumulation of gelatinous fluid [17]. Most recent data identified a premature stop codon in the *ADAMTS2* gene in these sheep [18]. The ADAMTS2 is an extracellular metalloproteinase involved in removal of propeptides of type I-III procollagens which is necessary for proper assembly of collagen fibrils in the extracellular matrix [19, 20].

A case of bovine dermatosparaxis analogous to human Ehlers-Danlos syndrome type VIIC and the ovine dermatosparaxis was found to be caused also by mutations in the procollagen I N-proteinase (pnPI) or *ADAMTS2* gene [21, 22]. As a consequence, severe skin fragility is a prominent feature of both Ehlers-Danlos syndrome type VIIC and bovine dermatosparaxis [20, 21]. Dermatosparaxis affecting a herd of Drakensberger cattle in South Africa was characterized by only mild skin pathology suggesting that this cattle carried a mutation in a site different from the one involved described by Colige et al. [21, 22].

A form of Marfan syndrome was also recognized in cattle [23–25]. The first report of a known case described aortic dilatation and aneurysm together with ocular abnormalities (microspherophakia, ectopia lensis, and lens opacities) and skeletal involvement characterized by long thin limbs, joint and tendon laxity, and postural kyphosis in several related calves [25]. Disruption of aortic elastic fibers and decreased incorporation of fibrillin in the extracellular matrix were identified by light microscopy [26, 27]. Similarly, Gigante et al. have found decrease in elastic fibers in all of the examined tissues (ligaments, periosteum, joint capsule and peripheral arteries) [28]. As in humans, mutations at different sites of bovine *FBN1* may be responsible for Marfan phenotype. For example, a mutation in one of the calcium-binding epidermal growth factor-like (cbEGF-like) domains of fibrillin-1 was responsible for the Marfan phenotype in cattle described by Singleton et al. [23]. Calcium binding by cbEGF-like domains contributes to proper conformation and stability of fibrillin-1 [28–30]. The mutations in *FBN1* lead to decrease in levels of functional fibrillin-1, and thus to increased levels of active TGFβ [28]. Hirano et al. detected a mutation on the post-furin cleavage site sequence of the C-terminal domain of fibrillin-1 [24].

Though standing somewhat apart from Ehlers-Danlos and Marfan syndromes, two other hereditary canine disorders are worth mentioning because of known underlying biochemical defects. Cutaneous mucinosis was described only recently in shar-pei dogs [31] and is characterized by hyaluronic acid accumulation limited to the dermis [32]. This gives shar-pei dogs their characteristic creased appearance consisting of thick skin folds on their heads. Increased hyaluronan synthase-2 expression is considered the culprit of this disorder [33]. The second disorder is primary lens luxation or isolated ectopia lensis afflicting mainly terrier breeds [34]. A mutation in a splice donor recognition site in regional candidate gene *ADAMTS17* has been recognized as the cause by Farias et al. [34]. Ectopia lensis is a recognized component of Marfan syndrome and Weill-Marchesani syndrome. Interestingly, Morales et al. have described truncating mutations in human *ADAMTS17* gene in a form of Weill-Marchesani syndrome in people with ocular abnormalities and short stature [35]. Mutations in one or more other *ADAMTS* genes causes Ehlers-Danlos syndrome VII C in people and dermatosparaxis in cattle and sheep (see above) [18, 21], and possibly a form Ehlers-Danlos in horses as well (see below) [36].

Hereditary equine regional dermal asthenia (HERDA), also known under the name of hyperelastosis cutis, was first described in Quarter horses (and later in other breeds) as a disease not dissimilar to cutaneous asthenia of dogs: it affects primarily skin which is hyperelastic, fragile and thin with atrophic scars [37–41]. Though the lesions occur mostly on the dorsum, legs are often involved as well [39]. Manifestations of the disease usually appear around 2 years of age when horses begin training, and saddling leads to skin trauma [42]. Gunson et al. noted fragmentation

and disorganization of collagen fibers both by light and electron microscopy, and suggested that a non-inflammatory degradation and phagocytosis of collagen are relevant in the pathogenesis of HERDA [38]. Other investigators have noted changes in the skin of horses with HERDA which confirmed the findings of Gunson: thin dermis with sparse, fragmented collagen in affected horses by light microscopy and variation in diameter of collagen fibrils by electron microscopy, fragile skin associated with poor wound healing [37, 39, 40]. Moreover, biomechanical analysis revealed lower tensile strength and modulus of elasticity of affected skin [42]. The only observation of tissue involvement other than skin was made by Ishikawa et al. who described the presence of varying diameters of collagen fibrils in tendons with high proportions of very small fibrils [43]. Whether HERDA is an autosomal dominant [37] or, more likely, an autosomal recessive disorder [39, 40, 42] is not quite clear. A homozygous missense mutation in the sixth residue of mature cyclopholin B protein was found in HERDA horses [43, 44]. Cyclophilin B (CypB or PPIB), a member of the peptidyl-propyl isomerase, acts as a chaperon involved in proper folding of collagens and in several other functions directed at modulating extracellular matrix. The *CYPB* mutation leads to substitution of glycine to arginine. The consequence is delayed folding of type I procollagen in the endoplasmic reticulum of HERDA horses, and a decrease in hydroxylysine and glucosyl-galactosyl hydroxylysine content of nascent collagen [43]. The ratio of pyridinoline to deoxypyridinoline is increased in HERDA horses and may serve as a marker for HERDA [45]. However, it is clear from the literature that not all HERDA horses have a mutation in the cyclophilin B gene [46].

Degenerative suspensory ligament desmitis (DSLD) is a chronic, debilitating disease affecting primarily Peruvian Pasos and Peruvian Paso crosses [47, 48]. However, many other breeds are afflicted as well [49, 50]. It tends to run in families though no clear pattern of heritability has been established. Likewise, the pathogenesis and biochemical defect are not well understood. DSLD is characterized by an insidious onset of bilateral or quadrilateral lameness without a history of trauma or performance related injury. Typically, horses present with one or more dropped fetlocks (fetlock is a metacarpophalangeal joint between the cannon bone and the pastern) (Fig. 14.1b). Fetlock effusion, static and dynamic hyperextension and degenerative joint disease are hallmarks on physical examination. Ultrasonography of affected suspensory ligaments reveals diffuse loss of echogenicity, and an irregular fiber pattern [48, 51–53]. Treatment for DSLD-affected horses is empirical and directed at minimizing musculoskeletal pain and providing support exercise for the suspensory apparatus [54]. Though until recently DSLD was considered a collagen disorder strictly limited to suspensory ligaments [47], our data show that it is a systemic disease involving tissues and organs with high content of connective tissue components. Abnormal accumulations of proteoglycans were identified not only in the suspensory ligaments, but also in the superficial and deep digital flexor tendons, patellar and nuchal ligaments, aorta, coronary arteries and sclerae of DSLD-affected horses. Unlike in HERDA, skin is not affected [49]. In mild or incipient cases proteoglycans are present only intracellularly, surrounding nuclei of tenocytes (Fig. 14.2b). However, with the progression of the disease this material is deposited extracellularly (Fig. 14.2c), and, with time, it displaces collagen fibers (in tendons) and elastic fibers (ligaments), and in many cases it is interspaced with newly formed cartilage foci (Fig. 14.2d). In light of these observations, a more appropriate term for this disease process may be equine systemic proteoglycan accumulation (ESPA) [49].

The occasional presence of whirls of active exuberant fibroblasts which contain very little collagen in tendons from DSLD-affected horses represents most likely an early stage of the disease. With time these foci eventually progress to a less cellular (and finally acellular) phase characterized by increasing proteoglycan content. Typically, no inflammatory or fibrotic changes accompany deposits of proteoglycans or proliferative lesions at any stage, early or late [49]. We hypothesize that the proliferating fibroblasts

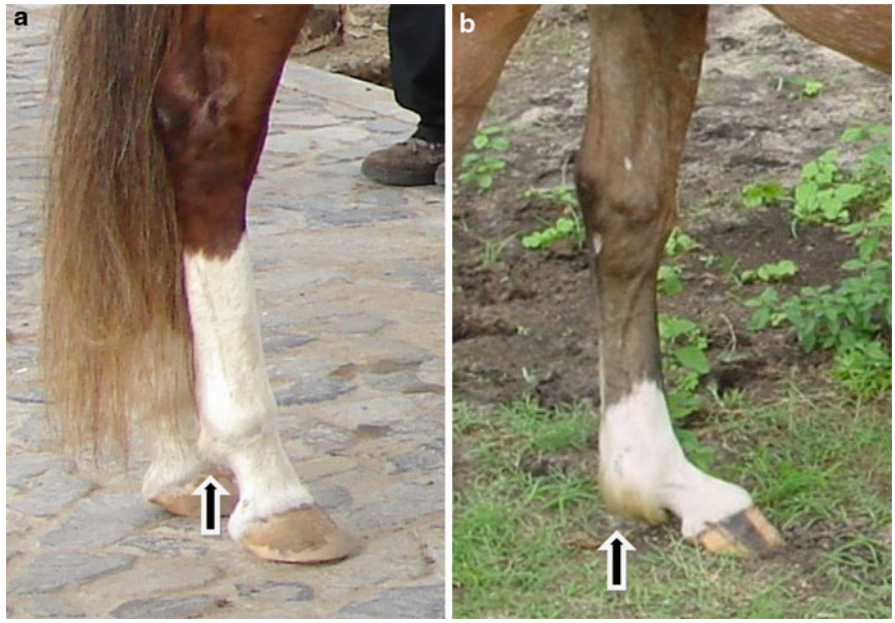

Fig. 14.1 (**a**) The photo shows the fetlock (metacarpophalangeal joint, ↑) of a back leg of a healthy Peruvian Paso horse. (**b**) This is in sharp contrast with the fetlock of a back leg of a DSLD-affected horse, note that the joint has dropped almost to the vground (↑). This is due to the weakness of the suspensory ligaments (the photos are courtesy of David and Laura Burrell)

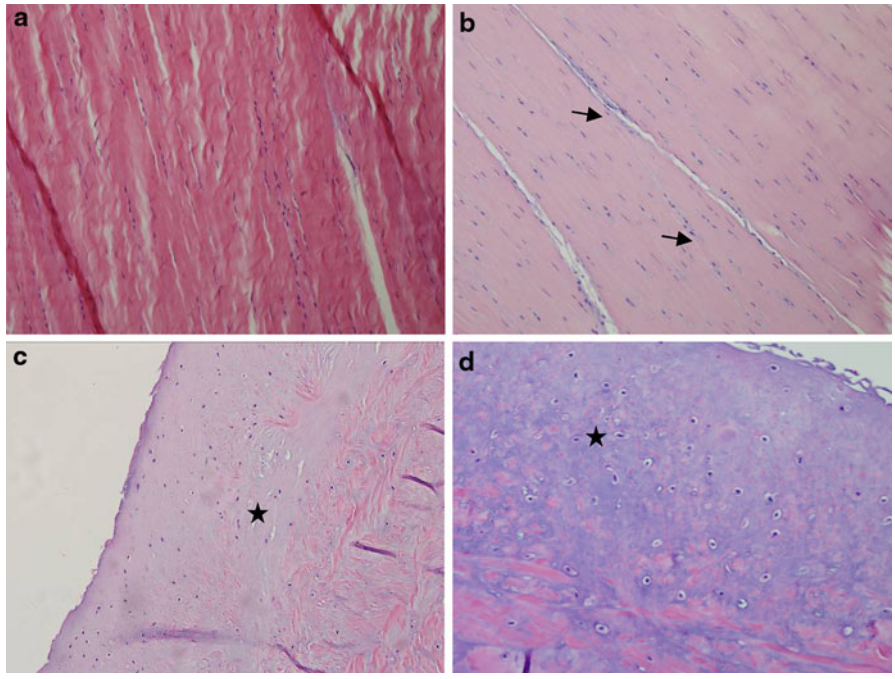

Fig. 14.2 (**a**) Control, healthy tendon stained with hematoxylin and eosin, original magnification ×200. (**b**) DSLD tissue, lightly infiltrated with proteoglycans (→), stained with hematoxylin and eosin, original magnification ×200. (**c**) DSLD tissue with heavy accumulation of proteoglycans (*) stained with hematoxylin and eosin. (**d**) Incipient cartilage formation in tendon heavily infiltrated with proteoglycans (*). Original magnification ×100 (portions of this figure originally appeared in Kim et al. [62])

secrete proteoglycans which, as the disease progresses, then accumulate in tissues. The stimulus for the proliferation of fibroblasts and the subsequent production of proteoglycans is unknown. Miller and Juzwiak have described a case of a 3 month old foal suffering from acute rupture of suspensory ligaments in the hindlimbs. On necropsy they found multifocal fibrovascular proliferation with only minimal inflammation and proteoglycan accumulation [55]. This would be similar to the proliferative lesions described above. Other investigators have also observed an increased accumulation of proteoglycans in suspensory ligaments and superficial and deep digital flexor tendons in DSLD, but not in other organs [36, 56], or they found lesions similar to those seen in DSLD but present only in few tissues or organs. For example, arterial medial calcification with elastin fiber disorganization in multiple arteries was described in a horse as an isolated phenomenon limited to the blood vessels [57]. However, it is not clear whether any tendons or ligaments were examined as well. In our experience calcifications within the blood vessel walls were rare in DSLD, though whether affected horses have propensity for sudden death due to coronary artery involvement or rupture of the aorta is a matter of some controversy [49]. Whether there is a relationship between DSLD and isolated aortic rupture syndrome occurring with higher frequency in Friesian horses remains to be seen [58–60], as these horses do not exhibit other problems. The presence of periaortic hemorrhages, media necrosis and fibrosis of the media and fibrosis of the adventitia of the aorta and pulmonary at least in some horses suggests the possibility of a soft tissue disorder akin to Marfan or Ehlers-Danlos [59].

Several aspects make the presence of cartilaginous metaplasia in the sclera of sheep as described by Smith et al. different from scleral involvement in horses with DSLD: no involvement of other tissues and organs, and high incidence of scrapies in these sheep [61]. In addition, we have not seen real cartilaginous metaplasia in any of the examined sclera in our cases so far.

The pathogenesis and underlying biochemical/genetic defect of DSLD are under investigation. Our data point to the presence of an abnormal form of decorin with altered biological activity in these proteoglycan deposits (Fig. 14.3). The abnormal form of decorin is the result of increased sulfation of at 6-position of N-acetylgalactosamine, and an increased ratio of glucuronic to iduronic acid in DSLD-affected tissues. As a consequence, chondroitin sulfate replaces dermatan sulfate at least partially during synthesis of the modified decorin [62].

Plaas et al. found abundant aggrecan content in proteoglycan accumulations of suspensory ligaments of DSLD-affected horses. This aggrecan appears to be of two types: one is a high molecular weight aggrecan identical to the form produced by equine mesenchymal cells rather similar to the form synthesized by articular cartilage. The other type consisted of aggrecan fragments generated by elevated levels of A-disintegrin-and-metalloproteinase-with-thrombospondin-like-motifs (ADAMTS) aggrecanase activity. The authors hypothesize that the high molecular species of aggrecan forms a high affinity substrate for ADAMTS5. They found elevated levels of ADAMTS5 and ADAMTS4 in these ligaments. Their findings bear similarity to findings in ADAMTS5 knockout TS5$^{-/-}$ mice [36]. TS5$^{-/-}$ mice have problems in skin wound healing [63] and develop biomechanically compromised tendons due to aggrecan accumulation in the pericellular matrix of fibroblasts [64]. In turn, as Wang et al. point out the aggrecan accumulation may lead to tendon dysfunction through impairment of TGFβ signaling ALK5/SMAD3 pathway [64]. ADAMTS5 cleavage of aggrecan occurs as one of numerous responses to TGFβ1stimulation of formation of connective tissue and fibrosis during physiological and pathological reparative processes, including perhaps even DSLD [36, 63]. The absence of ADAMTS5 (for example in TS5$^{-/-}$ mice) leads to assembly of pericellular CD-44 bound hyaluronan-aggrecan complexes into larger cell aggregates which disrupt repair of healing tissues such as dermis and tendons [63, 64]. This assembly is mediated by increased SMAD1/5/8 signaling as the absence of ADAMTS5 also disrupts TGFβ1 signaling pathways [63]. This finding would perhaps provide

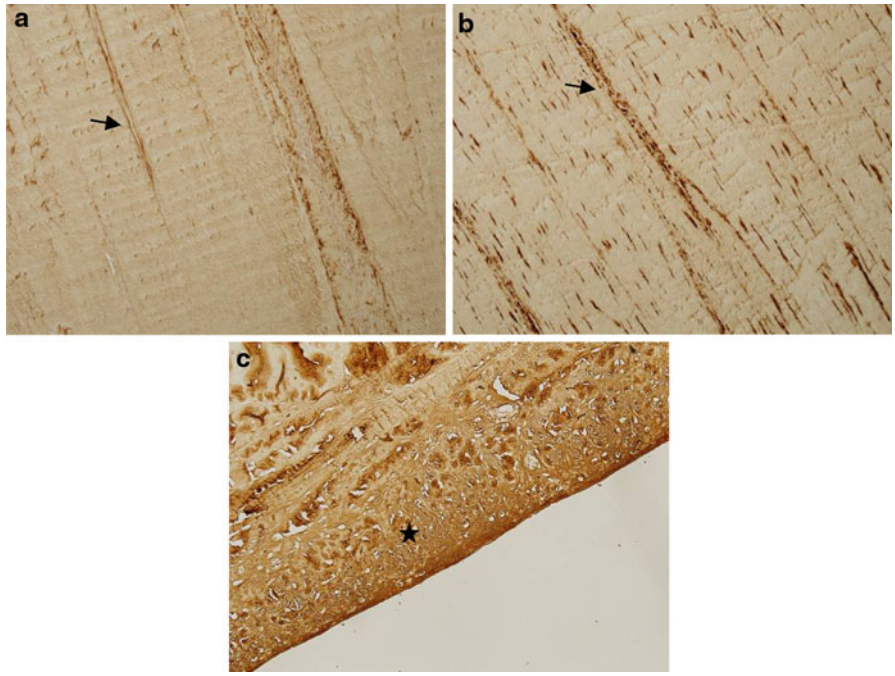

Fig. 14.3 Immunohistochemistry of superficial digital flexor tendon for decorin. Histological sections of superficial digital flexor tendons were immunostained with polyclonal rabbit LF122 antibody to decorin. Antigen–antibody complexes were detected with DAB staining. (**a**) Control tendon immunostained for decorin (→), original magnification ×200. (**b**) DSLD tendon, lightly infiltrated with proteoglycans, immunostained for decorin (→), original magnification ×200. (**c**) DSLD tendon with heavy accumulation of proteoglycans immunostained for decorin (*), original magnification ×100. Immunostaining for decorin in control tendons is more evenly distributed, and with less intensity in the healthy tendon than in DSLD-affected tendon (portions of this figure originally appeared in Kim et al. [62])

explanation for increased presence of TGFβ1 in DSLD tissues [62]. The alterations in TGFβ1 activity and function are reminiscent of TGFβ1 dysfunction in Marfan syndrome, and may lead to similar symptomatology and pathological findings in both DSLD (and EDS) and Marfan and Marfan-related syndromes, such as aortic rupture, myxomatous changes in cardiac valves (including, mitral valve prolapse).

Our most recent preliminary data also show alterations in aggrecan processing by ADAMTS5 in DSLD tissues (data not shown). In addition, increased levels of sulfatase 2, an enzyme involved in the removal of 6-*O*-sulfate groups from heparan sulfate [65], were found in DSLD tissues (data not shown).

Such naturally occurring diseases in domestic animals have a potential to serve as good models for analogous human diseases, and at times they might be better suited for this task than the commonly used rodent models. However, unless the pathogenesis and biochemistry of the syndromes and disorders are better defined and described for each species the usefulness of serving of these naturally occurring disorders as models for human disease will be limited. This is the case particularly relevant to dogs as a half out of the more than 400 naturally occurring hereditary canine diseases has the potential to serve as a model for human disease [66].

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Index

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