Am J Transl Res 2018;10(11):3291-3309
www.ajtr.org /ISSN:1943-8141/AJTR0079000

Review Article

Collagen I: a kingpin for rotator cuff tendon pathology

Finosh G Thankam, Matthew F Dilisio, Richard M Gross, Devendra K Agrawal

Department of Clinical and Translational Science and Orthopedic Surgery, School of Medicine, Creighton University, Omaha, NE 68178, USA

Received May 4, 2018; Accepted October 21, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: Derangements in tendon matrisome are pathognomonic for musculoskeletal disorders including rotator cuff tendinopathies (RCT). Collagen type-1 accounts for more than 85% of the dry weight of tendon extracellular matrix (ECM). The understanding of basic tendon physiology, organization of ECM, structure and function of component biomolecules of matrisome and the underlying regulatory mechanisms reveal the pathological events associated with RCT. Histomorphological evidence from RCT patients and animal models illustrate that ECM disorganization is the major hallmark in tendinopathy where a significant decrease in type-1 collagen is prevalent. However, the molecular events and regulatory signals associated with the regulation of collagen organization and its composition switch in response to pathological stimuli are largely unknown. The elucidation of various regulatory signalling pathways associated with collagen type-1 gene expression could benefit to develop novel promising therapeutic approaches to restore the tendon ECM. The major focus of the article is to critically evaluate tendon architecture regarding type-1 collagen, the molecular events associated with gene expression, secretion and maturation, the possible mechanisms of type-1 collagen regulation and its translational significance in RCT management.

Keywords: Rotator cuff tendinopathies, shoulder injury, type-1 collagen, tendon matrisome, tendon biology

Introduction

Tendinopathy accounts for about 50% of both work-related and sports-related injuries. The most vexing clinical issue associated with tendinopathies is its asymptomatic onset leading to tendon rupture [1]. Rotator cuff tendinopathy (RCT) is the most common tendon disorder of the shoulder which causes pain and dysfunction. Even though the multifactorial etiology of RCT is being studied, the biochemical and molecular events behind the pathogenesis remain unclear [2]. Rotator cuff tendinopathies can result from impingement, partial/full thickness tears, biceps tendinopathies, and frozen shoulders [3]. The understanding of basic biology, histology, molecular composition, and the associated signalling pathways are necessary in order to understand the pathological events associated with RCT. The current knowledge on the pathogenesis of RCT is almost entirely based on the findings in animal models and surgically removed tendons from patients and cadaver specimen [1].

Recently, we reported that the disorganization of tendon extracellular matrix (ECM) due to alteration of collagen composition and existence of inflammation without the active involvement of inflammatory cells are the major hallmarks of RCT [4-6]. The delay in restoring the altered Collagen I to III ratio was found to be another reason underlying the persistence of RCT [7]. In addition, increased cellular density, alterations in cellular morphology from spindle to round, apoptosis and necrosis have also been reported [4, 8]. Rotator cuff (RC) tendons being hypovascular rely mostly on the metabolome secreted from the local environment and their autocrine/paracrine effects to maintain the ECM homeostasis [10]. Moreover, the mechanical load exhibited by the tenocytes acts as a trigger to upregulate the expression of various mediators of remodeling and adaptation. However, in RCT the tenocytes fail to withstand the stress and the resulting impaired mechanobiologic-response affects the tendon homeostasis [9, 10, 13]. Remarkably, the altered tendon matrisome is the root cause for RCT [7] and the exploration of matrisome integrity and homeo-

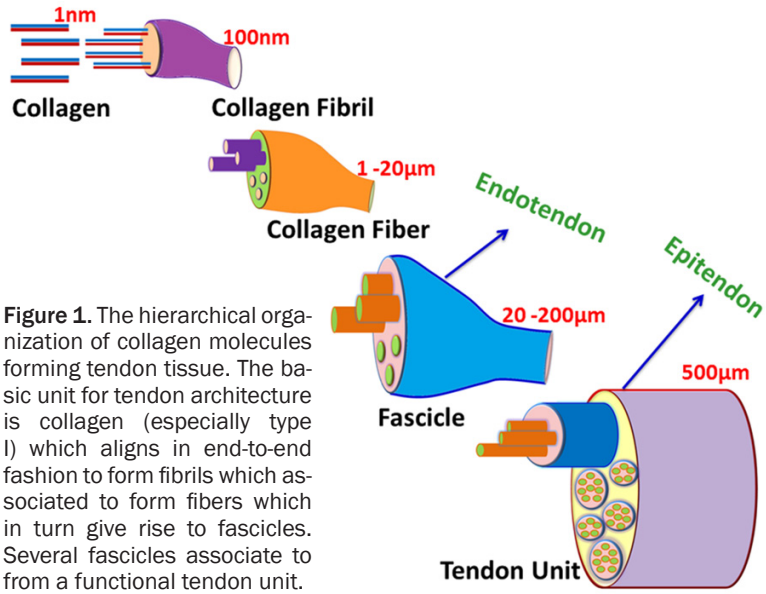


Figure 1. The hierarchical organization of collagen molecules forming tendon tissue. The basic unit for tendon architecture is collagen (especially type I) which aligns in end-to-end fashion to form fibrils which associated to form fibers which in turn give rise to fascicles. Several fascicles associate to from a functional tendon unit.

stasis in relation to RCT is the focus of this article.

Tendon ECM structure-the hierarchical organization

In its natural state, tendon tissue is composed of 70% water while more than 85% of the dry weight is constituted by collagen I [14]. The cells are limited and more than 90% of the available cells are fibroblasts [2, 15]. Since collagen I is the major component of tendon ECM, the structural and mechanical properties of tendon tissue depend largely on the quantity and quality of type I collagen. The hierarchical organization of collagen molecules is peculiar to tendon tissue. The collagen molecules associate to form fibrils, fibrils to fiber bundles, fiber bundles to fascicles and several fascicles constitute to form tendon units. The tendon units align in parallel to the long axis of tendon which is evident from basic histological staining. The collagen fibril is the basic structural unit of tendon and is formed by end-to-end organization of collagen type I (1 nm diameter) molecules. However, the fibril diameter varies from 10-500 nm depending on age, anatomical location, extent of physical activities and genetics. The usual trend is the increase in fibril diameter with age. Collagen fibrils constitute to form fibers (1-20 μm) which are enclosed by endotendons. Endotendons are thin connective tissues supplied with blood vessels, lymph vessels and nerves. The fibers organize to form fascicles (20-200 μm) where fascicle bundles are covered by epitendon. Similar to endotendon, epitendon surrounds a functional tendon unit (~500 μm) and is also supplied with blood, lymph and nerves [12]. Along with epitendon, paratendon also covers tendon tissue at certain sites. However, epitendon and paratendon make up to form peritendion which is responsible for minimizing the friction between the adjacent tissues [16]. The hierarchical organization of collagen molecules in the RC tendon tissues are displayed in **Figure 1**.

Biosynthesis and secretion of type I collagen-a key to RC tendon function

Type I collagen constitutes more than 95% of the total collagen content of RC tendon tissue, where the remaining 5% are mostly collagen type III and type V [14, 17]. Type III collagen is mostly concentrated to the regions of endotendon and epitendon and also in injured tendon [18, 19]. On the other hand, type V collagen intercalates with type I at the core and functions to regulate the fibril growth [20]. Besides, collagen type II, type VI, type IX and type XI also exist in minute levels but their exact function in RC tendon is largely unknown [21]. Type I collagen is the major collagen in RC tendon ECM and the major focus of tenocyte metabolism is to maintain matrisome integrity by regulating type I collagen.

Tenoblasts are the fibroblasts of tendon tissue which actively synthesize ECM components, especially collagen I [22]. Tenoblasts are the mechanoresponsive cells in post-natal tendon tissue which maintain tendon homeostasis by regulating ECM integrity [10]. The actual mechanism behind the synthesis and the regulatory events during conditions like embryonic development, regeneration, repair and inflammation are poorly known. However, the collagen molecules secreted by these cells attain a thermodynamically stable conformation by self-assembly [23]. The tenoblast cells secrete collagen precursor, the procollagen, as large aggre-

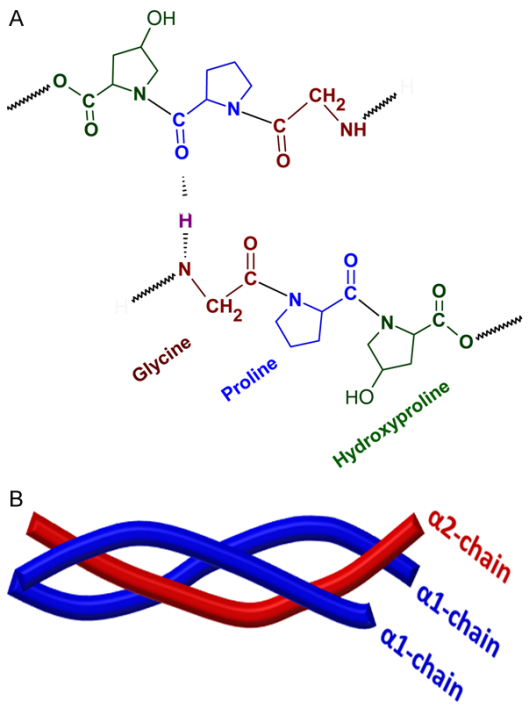


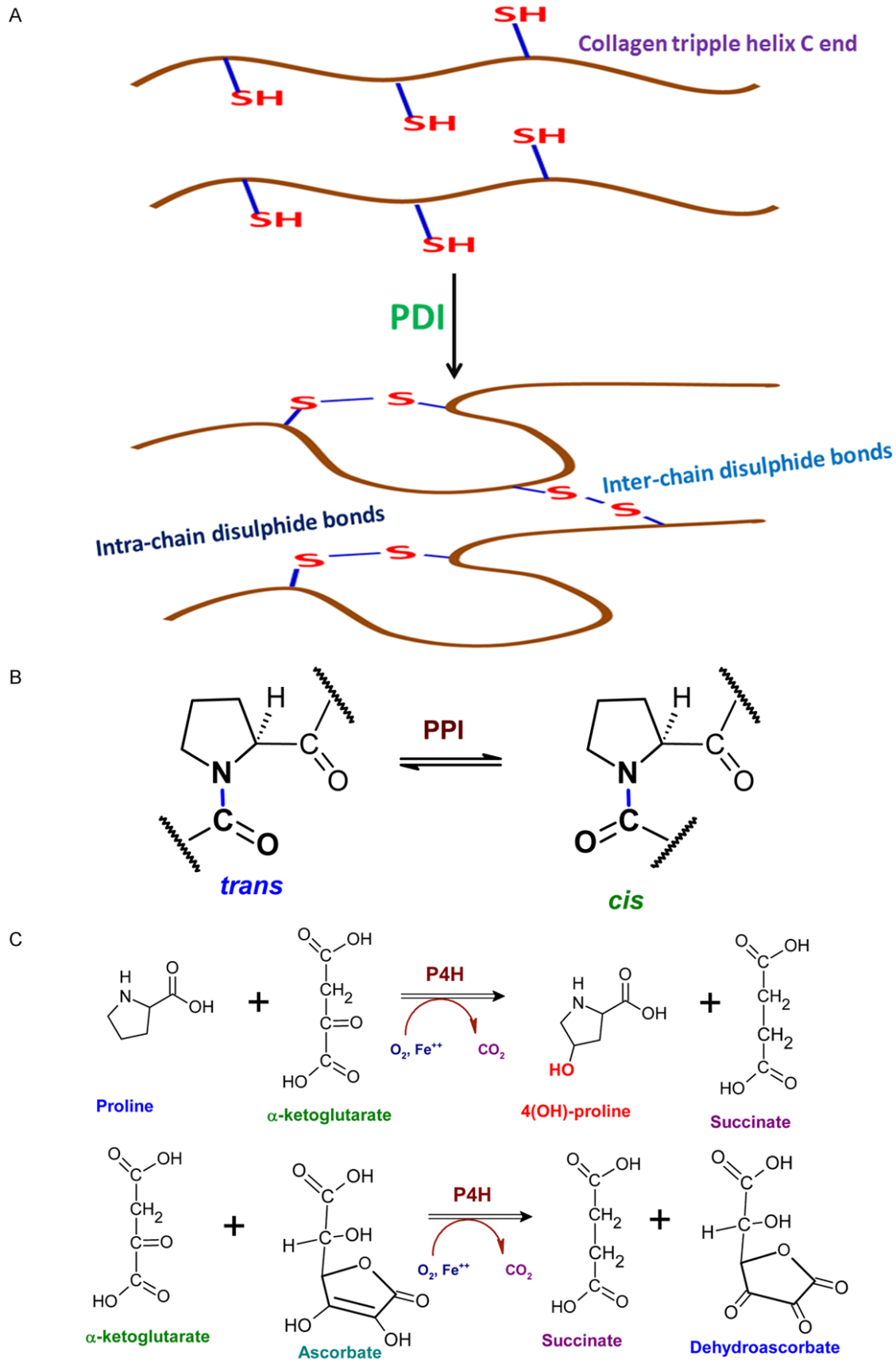
Figure 2. The molecular organization of collagen type I. A. The building units of collagen comprising repeating units of Gly-X-Y which is stabilized by hydrogen bonds. X and Y can be any amino acids, but usually Proline and Hydroxyproline respectively. B. The triple helical structure of collagen type I collagen comprising of two α-chains and one β-chain.

gates in the lumen of *cis*-Golgi complex. The large dimension of procollagen-aggregate creates a hurdle in loading them into small transport vehicles. It was elucidated that the *cis*-Golgi procollagen moves to *medial*-Golgi cisternae and then to *trans*-Golgi in a wavy fashion for secretion to ECM [24]. To date, there are more than 27 different types of collagen have been characterized where type I and III are significant for RC tendons [23].

All fibrillar collagen subtypes are made of repeating units of Gly-X-Y, where X and Y can be any amino acid residues, but usually Proline (Pro) and Hydroxyproline (HyPro), respectively (**Figure 2A**). The repeating triplet motif forms a left-handed helix which in turn organizes a right-handed triple helix by interacting with two other left-handed helices. The collagen molecules can be homotrimeric or heterotrimeric depending on the collagen subtype. For instance, collagen type I is a heterotrimer comprising two α-1 chains and one α-2 chain while type III collagen is made up of three α-2 chains (**Figure 2B**) [25]. The triple helical domain is 300 nm in length connecting N and C globular domains which do not necessarily follow Gly-X-Y repeating sequence. The proteolytic cleavage of N and C globular domains release collage triple helical molecule. The collagen units have short telopeptides on their both ends which assemble in an end-to-end fashion to form the fibril. The fibrils can be made up of type I and/or type III collagens in RC tendon tissue [26].

After synthesis, the procollagen type I translocates to ER (endoplasmic reticulum) lumen where the trimerization takes place, which is mediated by several molecular chaperones like HSP47 [26]. The procollagen contains propeptides at their C (carboxy-terminal propeptide of procollagen type I, PICP) and N (amino-terminal propeptide of procollagen type I, PINP) terminal ends which are proteolytically cleaved to integrate with the fibril. The cleaved fragments are released in the blood stream where the circulatory levels of the cleaved fragments represent the index of collagen synthesis. The 1:1 stoichiometry for PICP and PINP in the blood stream exist, but for PIIICP and PIIINP it may vary. This is because the proteolysis is incomplete during fibril formation, but completes during collagen type III degradation [29]. PICP is responsible for folding and chain selection for the nucleation/growth of fibrils. PICP facilitates the folding and collagen fibril formation by associating procollagen chains by disulphide bond formation within individual C propeptides before their cleavage. Protein disulphide isomerase (PDI) is the enzyme responsible for association of the procollagen chains with inter-chain disulphide bonds (**Figure 3A**). However, the directional folding from C to N terminal side also occurs independent of PDI reaction [23].

Additional modifications are required for maintaining the thermodynamic stability of the type I collagen triple helix. Peptidyl proline *cis-trans* isomerase (PPI) converts *cis*-Pro residues to *trans* isomers which is essential for proper folding and fitting into triple helix (**Figure 3B**) [30]. Moreover, the hydroxylation of Pro residues is required for the stability of triple helix as evident by the increased temperature for denaturation of collagen I with hydroxylated Pro residues. Prolyl-4-hydroxylase (P4H), a tetrameric enzyme with two-α and two-β subunits where the β subunit possess catalytic domain, cata-


A
SH
Collagen tripple helix C end
PDI
S
Inter-chain disulphide bonds
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B
PPI
trans
cis
C
Proline
α-ketoglutarate
P4H
O2, Fe++
CO2
4(OH)-proline
Succinate
α-ketoglutarate
Ascorbate
Succinate
Dehydroascorbate

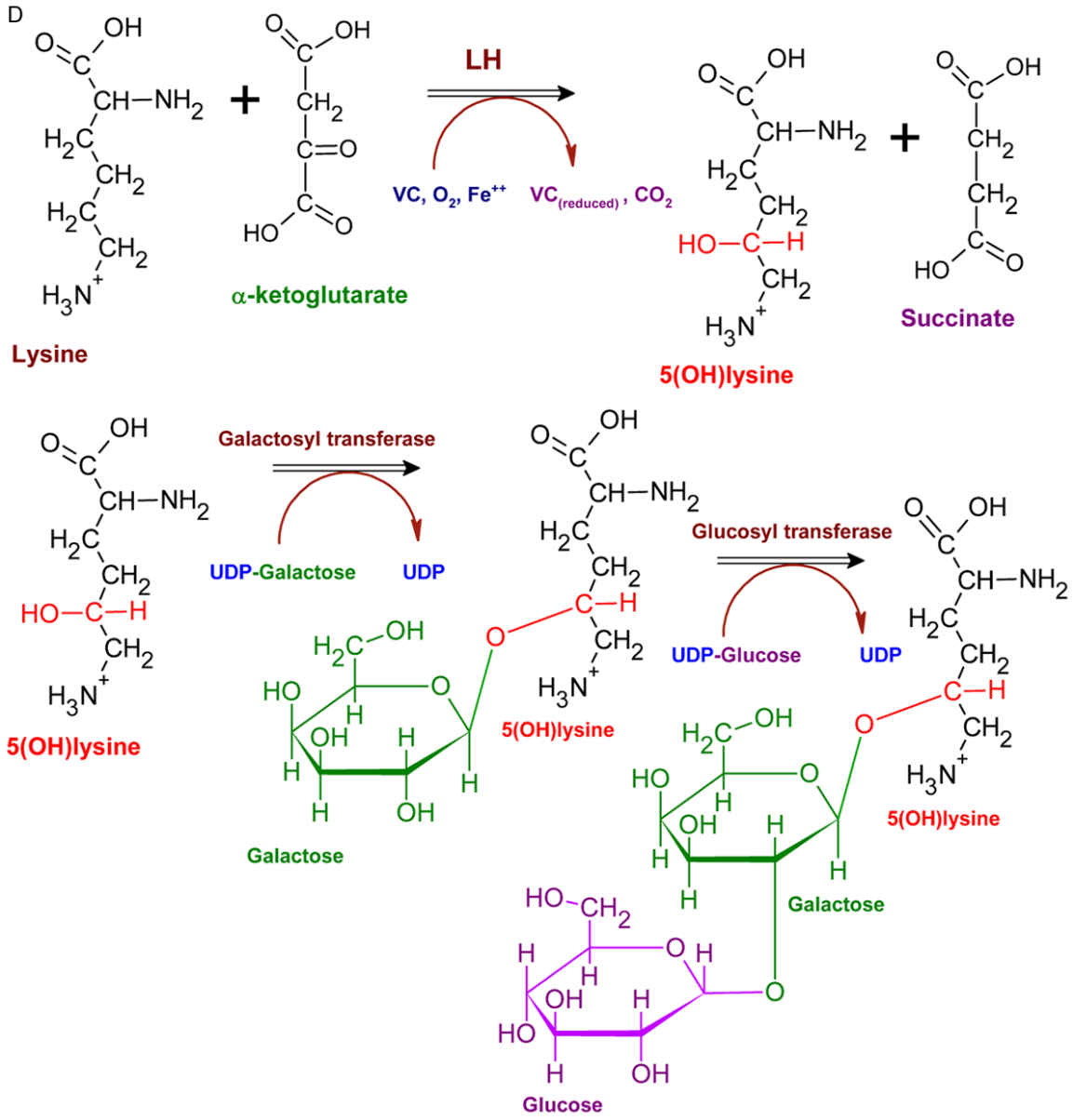

Figure 3. Enzyme activities associated with collagen type I formation. A. PDI catalyzes the formation of inter-chain and intra-chain disulphide bonds especially at the C terminal end of procollagen which is inevitable for the proper organization of triple helix. B. PPI converts *cis*-Pro residues to *trans* isomers which is essential for proper folding and fitting into triple helix. C. P4H is an iron-containing enzyme responsible for hydroxylation of Pro residues. Oxygen, proline residue, and α-ketoglutarate interact with the enzyme and the proline residue is hydroxylated while the α-ketoglutarate undergoes oxidative decarboxylation to form succinate and carbon dioxide. The role of vitamin C is shown as another reaction which prolyl 4-hydroxylase catalyzes the oxidative decarboxylation of α-ketoglutarate. But, in this reaction the iron is oxidized which inactivates enzyme. Vitamin C (ascorbate) reduces the iron at the vicinity of the enzyme to restore its active state. D. LH catalyzes the hydroxylation of Lys residues at the 5th C atom to from 5(OH)lysine and LH acts like P4H enzyme. The 5(OH)lysine acts as a template for the addition of galactose moiety followed by glucose moiety to the procollagen by the hydroxylysl galactose transferase and galactosyl hydroxylysl glucose transferase respectively. UDP-Galactose/Glucose acts as sugar donors and this reaction is crucial for trafficking of procollagen from Golgi to ECM.

lyzes the Pro hydroxylation reaction. The β subunit possesses PDI activity also. The enzyme requires vitamin C (VC) for its activity which depends on high cell density to induce collagen pathway (**Figure 3C**). The cell density stabilizes the effect of triple helix in regard to the chang-

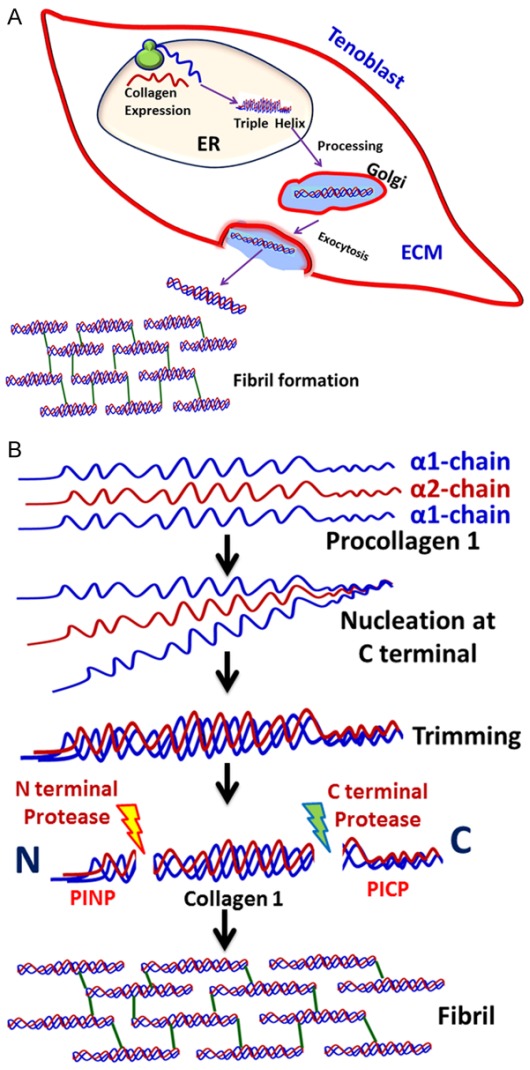

Figure 4. Secretion of fibrils to ECM in tenoblasts. A. The collagen gene expression and assembly of triple helix occurs in the ER lumen of tenoblasts. The procollagen is then processed in Golgi apparatus and is transported to plasma membrane for exocytosis of mature collagen to ECM where the fibril formation takes place. B. The molecular events in the assembly of fibril. The procollagen type I subunits were synthesized in the ER lumen which align to form triple helix by nucleating at the C terminal end by disulphide formation. The collagen triple helix matures by the trimming of PICP and PINP by corresponding proteases. This triple helix aligns at end-to-end fashion in the ECM to form fibrils.

es in cellular redox potential [31]. Hydroxylation holds an extensive network of water molecules within the triple helix by coordinately bridging them within and between the chains [23]. Similar sort of hydroxylation occurs in Lys residues as well. The added -OH groups act as cues for attaching galactose and glucose moieties in the ER by specific transferases (**Figure 3D**) [23].

The glycosylation of procollagens occurs in ER and Golgi complex. Galactose and glucose residues added to the hydroxylysine and the oligosaccharide units added to Asn residues at the C terminal end of procollagen is required for proper alignment for its transport to ECM. Usually the hydroxylation of Pro and Lys residues in the middle portion are hydroxylated by membrane bound hydroxylases. Once these events are completed, the type I procollagen is secreted to extracellular space by exocytosis. In the extracellular space, the N and C terminal propeptides are removed by procollagen peptidases resulting in tropocollagen, which consists of mostly triple helix. The N and C terminal trimmed procollagen polymerize to form fibrils in the extracellular space. This assembly is accelerated by lysyl oxidase, an extracellular enzyme which creates aldehyde groups in Lys and contributes to crosslinking and fibril stability. The maturation of collagen molecules requires additional post-translational modifications [24]. The molecular and cellular events associated with collagen fibril formation by tenoblast/tenocytes are depicted in **Figure 4**.

The procollagen type I protein needs to attain its natively folded conformation to reside in the ER. The loading of cargo (here procollagen type 1) is under the control of two proteins COPI and COPII (coat protein complex I and II). COPI and COPII coat the outer surface of transport vesicles [32]. The fibrils being ~300 nm diameter and the 60-90 nm vesicles suggest the possibilities of alternate carriers for lager cargos like collagen type I [23]. Evidence from freeze fracture technique, light/video electron microscopy and GFP-tagging experiments revealed the transport of procollagen as saccular structure budded from ER membrane and the ECM-targeted proteins are segregated from other targets in early stage [29-31]. The COPII vesicles are necessary for the export to procollagen type I loaded cargo at the ER exit site.

COPII coat assembly requires six polypeptides. Initially, GDP-bound Sar1 (Secretion-associated RAS-related protein 1) polypeptide interacts with Sec12 and exchange its GDP with GTP [36]. This activates Sar1-GTP to insert its hydrophobic domain to ER outer-membrane where it recruits and binds with Sec23/Sec24 pro-

tein coated complex. The binding is between Sec24 and GTP-bound Sar1. This assembly forms the first layer of coat protein in the cytoplasmic face of ER. Binding of Sec24 facilitates the cargo to COPII in the transmembrane through interactions with hydrophobic, aromatic and acidic motifs [37]. COPII pre-budding complex then recruits Sec13/Sec31 dimer and Sec31 directly interacts with Sec23 via a proline-rich domain. Sec23 also serves as a GTPase activation protein (GAP) for Sar1 and the GAP activity is regulated by the interaction of Sec31 through the proline-rich domain. The two events, Sar1 GTPase activity and outer coat layer recruitment, complete the biogenesis of COPII and trigger the budding. These coat proteins are highly conserved in all eukaryotes [38].

Transport ANd Golgi Organization 1 (TANGO1) protein (1907 amino acids) is also reported to be involved in the trafficking of larger cargoes like collagen fibrils to exit ER. This is evident from the silencing experimental data on HeLa cells which confirmed that the depletion of TANGO1 significantly affected collagen type VII without affecting general protein secretion [35, 36]. Interestingly, type 1 collagen was also affected in TANGO1 knockout mice [41]. However, TANGO1 plays a role in synchronizing the sorting of type I collagen from other types [42]. TANGO1 resides at the exit site of ER and is comprised of SR3-like domain and a coiled-coil domain at N-terminus, a cytoplasmic domain and a C-terminus Pro-rich domain. TANGO1 bears two closely located hydrophobic domains; a transmembrane domain and a partially embedded domain in the outer or inner leaflet [40]. Collagen VII binding to SH3 domain of TANGO1 in ER lumen facilitates the binding of Sec23/Sec24 via Pro-rich domain. This binding blocks the Pro-rich domain-mediated binding of Sec31 to Sec23 and delays Sar1-GTP hydrolysis. This temporary block provides enough time for COPII to accommodate larger cargoes like collagen fibrils. After loading to COPII, the collagen molecule dissociates from TANGO1 which couples the dissociation of TANGO1 from Sec23/Sec24. Now, Sec23/Sec24 is exposed for receiving Sec13/Sec31 to trigger budding. In short, TANGO1 provides enough time to load larger cargos to COPII carrier. Another protein, cTAGE5, binds to TANGO1 Sec23/Sec24 to facilitate effective packing of fibrils [34, 36].

Defects in the ER transport of collagen type I fibrils can affect the integrity of RC tendon ECM and lead to poor quality and low quantity collagens. But, the studies relating ER defects and RC tendon disorders are rare in the literature. However, the active role of ER proteins in the collagen metabolism is clearly understood [43]. Moreover, the age-related alterations in ER transport of collagen fibrils has been demonstrated in horse tendon [44]. Since RCT are characterized by alterations in the collagen ratio, the possibilities of ER defects should not be overlooked. Extensive research is needed in these aspects on RC tenoblasts/tenocytes which would have translational significance in RCT management.

The transport of collagen cargo in the anterograde direction across the Golgi apparatus of mammalian cells is largely unknown and is still under debate. The cisternae of Golgi apparatus can mediate sequential post-translational modifications of procollagen type I molecules [38]. The glycosylation enzymes are in the cisternae where the COP coated vesicles are associated. Interestingly, the procollagen can move across the Golgi without being detached from cisternae. The maturation of cisternae results in the retrograde transport of local enzymes especially mannosidase I and sialyl-transferase [23]. Once budded from ER exit site, they shed the coat and undergo homotypic (between same compartments) or heterotypic fusion (between different compartments). The SNARE [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors] proteins especially, v-SNAREs (Vesicle-SNSAREs) and t-SNAREs (target-localized SNAREs) aid in the fusion leading to the formation of continuous compartment in ER [24].

Evidence from electron microscopy of fibroblast revealed the release of procollagen fibrils from Golgi apparatus as secretory vacuoles [23]. The procollagen cargo from trans-Golgi-network (TGN) is transported to endosomes and lysosomes in a process mediated by clathrin-coated vesicles [45]. Clathrin-independent endosomal transport system and Ca^{2+}-based sorting of secretory cargo have also been reported [46]. But, the underlying mechanism and transport to the plasma membrane of procollagen is unknown. However, the involvement of protein kinase C-δ (PKCδ) in collagen type I export has

been identified in smooth muscle cells [47]. Still, the mechanism of PKCδ activation and the effect of PKCδ system in tendon cells are yet to be discovered.

Interestingly, the cross section of tendon tissues revealed the existence of collagen fibrils in the cytoplasm surrounded by cell membrane. This finding revealed the presence of cellular recesses for the secretion of collagen [48]. The single fibrils released through the recesses assemble to form higher order structures thereby contributing to tendon growth. The long collagen fibrils co-exist with several short ones within the cell and are referred as fibripositor (fibril-depositor). Fibripositor extends from the side of tenoblasts; align through the long axis of tendon and projects out to ECM through recesses. The carriers push the fibril out of the cell and subsequently fuse with the plasma membrane to form a new fibripositor. It can also fuse with the existing fibripositor. The procollagen molecules are added to the base of fibripositor facilitating the end-to-end fusion to form fibrils [49].

ECM processing of procollagen I-the final make-up

The proteolytic removal of globular C and N terminal peptides from procollagen type I limits the effective concentration required for fibril formation which in turn triggers self-assembly. The cleavage occurs during or following secretion to ECM. The enzymatic cleavage is performed by type-specific MMPs (metalloproteinases), like ADAMTS (A disintegrin and metalloproteinase with thrombospondin motif) especially ADAMTS-2, ADAMTS-3 and ADAMTS13, bone morphogenetic protein 1 (BMP1) and tolloid-like families like furin-like proprotein convertases (FLPC) [50]. The presence of C-propeptide increases the solubility of procollagen in ECM and prevents immature fibril formation. On the other hand, the N-propeptide directs the diameter and shape and contributes to increase in surface-to-volume ratio [50]. N-propeptides are completely removed from type I, II and III procollagens where the cleavage is rapid in types I and II but this process is relatively slow in type III. The delay in the removal of N-terminal propeptide accumulates partially-processed procollagen called pN-collagen (without C-propeptides with N-propeptides). The retention of N-propeptides in pN-collagen prevents its access to the center of fibril. Whereas, N-propeptides cause the incorporation of type III collagen to the surface and thus limits the fibril diameter. That is why, collagen III is usually associated with the surface of type I [51].

Electron microscopy data revealed the collagen self-assembly with a quarter stagger axial D periodicity of approximately 67 nm which appear as characteristic striations in the ECM images [50]. Micro-fibrils are formed by the sequence of events including nucleation, organization and elongation towards the long axis of tendon. It is believed that the N-terminal propeptides of type V are required for the occurrence of nucleation of type I and II collagens as these projects to the gap between adjacent fibrils and prevents the lateral growth by enhancing steric hindrance and charge interactions [52]. Fibril-associated collagens with interrupted triple helices (FACIT) like type XII and XIV (non-fibrillar collagen) were also reported to have a regulatory role in fibril formation. These non-fibrillar collagens aid in orchestrating matrix components with type I collagens by interacting with proteoglycans, fibromodulin, decorin and others [49, 50]. Rentz and colleagues reported the association of adhesive protein SPARC (secreted protein acidic and rich in cysteine) in the processing and incorporation of procollagen type I in tendons [51, 52]. Indeed, there is evidence for the involvement of several proteins and other biomolecules in the maturation of collagen type I in RC tendon. The functions of some of such molecules are given in **Table 1**. In a nut shell, the collagen fibril assembly owes to unique chemical and physical interactions resulting in a multi-hierarchical architecture which maintains the integrity of ECM in the RC tendon.

Matrisome composed of thousands of proteins and their co-ordinated interactions that are needed to sustain the ECM homeostasis. According to human matrisome database (HMD), 1139 proteins were associated with ECM, of which 128 proteins attribute solely to human ECM where 76 proteins associate with core matrisome and 52 proteins with ECM-associated components. Twelve proteoglycans, 18 collagens, and 46 glycoproteins comprise to form the core. ECM-associated components are constituted by 16 ECM-affiliated compo-

Table 1. Non-collagenous biomolecules associated with RC tendon matrisome

Biomolecules	Function	References
Small Leucine-Rich Proteoglycans (SLRPs)	Regulation of fibrillogenesis	[105]
Decorin	Mediator of fibrillogenesis by inhibiting fibril formation by lateral fusion, contribute to alignment, stability, strength and elasticity to collagen fibrils via surface interactions	[106]
Biglycan	Regulation of fibrillogenesis during development	[107]
Fibromodulin	Regulation of fibril fusion by inhibiting lateral fusion	[108]
Lumican	Regulation of fibril fusion	[109]
Cartilage oligomeric matrix protein (COMP)	Enhancer of collagen fibrillogenesis, mediates interaction between different components of ECM	[110]
Elastin	Stretch and recoil	[12]
Fibronectin	Cell adhesion	[111]
Laminins	Cell differentiation and migration	[112]
Tenascins	Load bearing and regeneration	[113]
Growth factors	Growth, repair and regeneration	[114]
MMPs	Repair and regeneration	[115]

Table 2. Regulators of collagen type I gene expression

Proteins	Function	References
CCAAT binding factor (CBF)	Interacts with CCAAT box located at -100 to -96 bp and enhance collagen I expression.	[116]
Nuclear factor-1	Binds between -350 and -300 bp of COL1A2 and act as a positive TF.	[117]
Inhibitor *protein 1 & 2*	Competes with CBF for binding to promoter and inhibits collagen I expression.	[59]
CCAAT/enhancer binding proteins	Basal and cytokine modulated Type I collagen gene expression.	[59]
Specificity factor 1 (SP1)	A zinc-finger family transcription factor that binds to GC-rich regions located between -303 and -271 bp in COL1A2 gene promoter and modulate collagen I expression.	[71]
Activator protein 1 (AP1)	AP1 binds at +292 to +670-bp region of first intron of human COL1A1 and functions in transcriptional regulation.	[118]
NF-κβ	Inhibits COL1A1 and COL1A2 expression.	[76]
Smad7	Smad binding element, CAGACA, is located at -263 to -258 bp, and Smad7 mediates COL1A2 gene transcription.	[59]
p300/CBP	p300/CBP are adaptor with intrinsic HAT activity that modulates Type I collagen gene transcription.	[119]
Myb	Modulation of Type I collagen gene expression in cell type dependent manner.	[120]
c-Myc	Suppress Type I collagen transcription.	[121]
c-Krox	Regulator of COL1A1.	[59]
Basic transcription element binding protein	Enhance COL1A1 expression.	[122]
Protease nexin 1 (PN1)	PN-1 increases the level of collagen transcription and decreases its degradation.	[123]
Protein phosphatase 2A	Enhance COL1A1 expression.	[124]
Extracellular signal-regulated kinase 1/2	Represses Type I collagen synthesis.	[59]
CBFA1	Transcription factor for osteoblast-specific gene expression of Type I collagen.	[125]
Connective tissue growth factor	Fibroblast proliferation and wound healing by stimulation of Type I collagen gene expression.	[126]

nents, 24 regulators and 12 secreted factors [57]. However, the proteomics of RC tendon matrisome has not been reported yet. Recently, we have reported some of the key protein mediators of RC tendon matrisome in regard to the miRNA mediated regulation of glenohumeral arthritis [7]. The identification of regulatory proteins of RC tendon ECM and the knowledge of their alterations associated with RCT could have translational impact to pave ways for the development of novel therapeutic strategies.

Collagen type I gene expression-a felicitous point for tendon ECM regulation

Human pro-COL1A1 (18 kb) and pro-COL1A2 (38 kb) genes are located in 17q21.31-22.05 and in 7q21.3-22.1, respectively [58]. Even though both sub-units (COL1A1 and COL1A2) are found on different chromosomal locations, the transcripts of these two genes are maintained as 2:1 ratio at the cellular levels. Both the genes contain several repressor and enhancer elements in their promoter regions, and binding sites for basal, cell-specific and cytokine-mediated transcription factors. Moreover, the cytokine-responsive elements (CREs) have also been identified and located in several tissues. Epigenetic regulation by DNA methylation and subsequent downregulation of type I collagen genes were also described [59]. The gene expression of collagen type I in RC tendons is also under strict regulation. However, it should be noted that the regulatory signals alter significantly depending on the type and severity of RCT. Since collagen I is the major component of RC tendon, the regulatory mediators mainly focus on the restoration of collagen I after RCT. Several regulatory molecules are associated with collagen type I gene expression and the functions of the common ones are shown in **Table 2**.

Since hypoxia is often associated with RCT, the hypoxic signaling elicited by the activation of hypoxia inducible factor (HIF) plays a crucial role in collagen homeostasis. The effect of HIF in collagen cross linking can be attributed to its action on lysyl oxidase (LOX) gene. HIF-1 and HIF-2 upregulate LOX where the effect of HIF-2 is superior due to its effective binding with the hypoxia response elements (HRE) of LOX promoter [60]. However, HIF-1α has been reported to upregulate collagen genes by activating NOTCH1, NOTCH1 ligand (JAGGED1) and hairy and enhancer of split-1 (*HES1*) [61]. There are four NOTCH (1-4) receptors and their corresponding ligands Delta-like (1-4) and JAGGED (JAG1 and 2) have been characterized, so far, in mammalian system. On binding with ligand the presenilin proteases (PSEN1/2) (part of γ-secretase complex, GSC) cleave intracellular domain of NOTCH (Notch intracellular domain, NICD) which translocates to nucleus and interacts with immunoglobulin κ J region (RBP-Jκ) and Mastermind-like (Maml) proteins which in turn trigger the transcription of *HES* and *HEY* (mammalian counterparts of drosophila *HES1*) genes [58, 59]. RBP-Jκ, also known as CSL (CBF-1, Su(H), Lag-1), is responsible for the binding of the complex to DNA. Interestingly, the NICD hyperactivity inhibits differentiation of osteoblasts by controlling collagen type 1 [64]. However, the effect of NOTCH signaling and its activation by hypoxia in RCT have not been studied yet; which opens up tremendous research opportunities especially in tendon matrix biology.

Growth factors (GFs) are another class of mediators that have active role in tendon matrix homeostasis, thereby play significant role in healing after RCT. The involvement of several GFs and their specific functions have been characterized in tendon tissues (**Table 3**). Among them, TGF-β signaling pathway has direct impact on tendon ECM regeneration which proceeds through a class of intracellular signaling proteins called Smads. Upon binding to its receptor TGF-β activates downstream Smad2 and Smad3 and facilitates to form heteromers by binding to the co-Smad, Smad4. This Smad complex translocates to nucleus and regulates the expression of ECM-associated genes, especially Col1A1. The hyperactivity of Smad complex is controlled by inhibitory Smads (I-Smads), which are Smad6 and Smad7. I-Smads interfere Smad2 and Smad3 phosphorylation thereby block the interaction of TGF-β receptors (negative-feedback) [65]. Moreover, TGF-β activator protein (TAP) binds to TGF-β *cis*-element and the TGF-β-responsive sequences (TBRS) (between -174 and -84 bp) of downstream transcription start site of Col1A1 gene. In addition, TGF-β_1 induces type 1 collagen gene expression by the epigenetic inhibition of DNMT1, DNMT3a and global DNMT activity which result in DNA demethylation of the COL1A1 promoter

Table 3. Role of growth factors in RCT

Growth factor	Function	References
PDGF	Secretion of other GFs Cell proliferation Tendon healing DNA synthesis	[127]
TGF-β	Tendon healing Tendon matrix deposition Scarring and fibrosis	[12]
VEGF	Endothelial cell proliferation Angiogenesis	[128]
bFGF	Tendon cell migration and proliferation Angiogenesis Collagen type 1 gene expression	[12]
BMPs	Tenogenesis (BMP-12) Tendon callus formation (BMP-13 & 14) Mechanical strength Tenoblast proliferation Collagen type 1 and type 3 gene expression	[129]
IGF1	Mechanical strength	[130]

[66]. There are reports projecting the operation of TGF-β signaling in tendon tissue [67, 12]. However, the translational aspects of TGF-β with respect to COL1 gene expression in RCT are limited in the literature.

Most inflammatory signals converge to the transcription factor NF-κB which upregulate a battery of pro-inflammatory genes [68]. However, NF-κB downregulates *COL1A1* by interfering the activities of zinc fingers, Sp1 (specific protein-1) and Sp3 which act as *trans*-activators of *COL1A1* at an upstream 51 bp region (-112/-61). The p65 (RelA) subunit of NF-κB is responsible for the *trans*-inhibiting effect [69]. TGF-β-responsive activator sequences located 174 and 84 bp upstream from the initiation site of *COL1A1* have binding sites for Sp1 and NF-1 (nuclear factor-1) [70]. Similar effect of TGF-β *trans*-activation of *COL1A2* has also been established which confirms the role of TGF-β in ECM remodeling by upregulating COL1 synthesis [65, 67]. Apart from Sp, zinc finger proteins like c-Krox were also reported to upregulate *COL1A1* by -112/-61 upstream sequence [72].

NF-κB associated transcription factors share a conserved domain of ~300 N-terminal amino acids called Rel homology domain (RHD) which bears NLS (nuclear localization sequence). RHD facilitates DNA binding, dimerization and association with inhibitory IκB proteins. However, p65 subunit possesses C-terminal *trans*-activation domain for eliciting the transcriptional activities [69]. NF-κB is associated with IκB family of inhibitors in unchallenged cells thereby sequestering in the cytoplasm. It has been estimated that more than 200 signals can elicit the activation of NF-κB by inducing IκB dissociation which exposes the NLS permitting their nuclear entry and promoter binding. These signals include IL-1, IL-6, TNF-α, TREM-1 and others [65, 69, 70]. However, the release of NF-κB by IκB degradation is not sufficient for eliciting the transcriptional control but, requires other factors like Sp1. This explains NF-κB-mediated downregulation of *COL1A1* due to its interaction with Sp1 [75]. The p65 subunit also has an inhibitory effect on collagen gene expression [69].

TNF-α elicits the regulatory effects on *COL1A1* and *COL1A2* in fibroblasts and other cell types which is mediated by NF-κB family of transcription factors [72, 73]. TNF-α-inhibitory responsive element (TαRE) has been mapped in the upstream promoter region between 378 and 345 of *COL1A1* which host the binding site for a school of DNA binding complexes like p20C/EBPβ, p35C/EBPβ, and C/EBPδ. Interestingly, this sequence co-localizes with TGF-β-responsive element suggesting that the inhibitory effects of TNF-α proceeds by interfering TGF-β-induced activation, nuclear transport and DNA binding effects of Smad complexes [78]. Also, the inhibitory Smad7 has been reported to be activated by p65-mediated activation of TNF-α in rat hepatic stellate cells [79]. Thus, TNF-α signaling converges to NF-κB activation and shuts down COL1 synthesis. The operation of TNF-α and NF-κB signaling for collagen gene regulation has not been well established in tendon tissues, however, the elevation of these signals with a concomitant downregulation of collagen type 1 has been found to be associated with mechanically stressed tendon [80].

The prevalence of RCT in elder population is higher than in the younger subjects which often coincide with inflammatory (such as glenohumeral arthritis) or metabolic disorders (such as

Table 4. Major categories of MMP promoters, their features and function

Groups	Features	Function
Group I	TATA box ~-30 bp AP-1 binding site ~-70 bp PEA3 binding site adjacent to that of AP1	Regulates most MMPs
Group II	TATA box present AP-1 binding site absent	Regulates MMP-8, MMP11 and MMP-21
Group III	No TATA box Multiple start site for transcription Requires SP-1 family of TF Expression mediates through GC box	Regulates MMP-2, MMP14 and MMP-28

diabetes) [77, 78]. The etiology of RCT and collagen regulation associated with aging has not been elucidated yet. However, greater significance has been gained to the concomitant increase in advanced glycation end products (AGEs) and its implication to age-associated structural alterations in the ECM of several tissues. AGE signaling with its receptor RAGE also converges to NF-κB and subsequent inflammation. However, based on the type of signals NF-κB elicits different transcriptional outputs. This can be explained by 'NF-κB barcode hypothesis' which states that depending on dynamics of cellular network signal-specific post-translational modifications, "the barcodes" to NF-κB are induced which act as a signature for the specific gene expression pattern [83].

Apart from AGEs, RAGE shares a multitude of ligand subset which includes high mobility group box-1 (HMGB1), S100 family, and lipopolysaccharide (LPS) [80-82]. The expression of RAGE was also found to be enhanced with aging which has a strong correlation with diabetes and hyperglycemia [87]. In the present context, the collagen I and III expression have been found to be decreased with concomitant increase of RAGE in the arterial wall of RAGE null mice suggesting its effect on collagen production [88]. However, contrasting results were obtained in fibroblasts from different tissue sources [65, 85]. Recently, we reported that in the RC tendon tissues the decrease in the ratio of collagen 1 to 3 was a major cause for ECM disorganization [7]. Interestingly, we found an upregulation of RAGE/HMGB1 axis with a proportionate increase in TREM1 expression [4]. On correlating the increased TREM1 with decreased COL1 expression, we speculate that TREM1-mediated NF-κB activation proceeds through a different barcode other than RelA which warrants further investigation.

Metalloproteinase activity influences the expression of collagen types in RC tendon tissues following injury. Like other tissues, tendons also have a basal level of MMPs under normal conditions where a rapid upregulation occurs immediately after the injury. Most cell types are capable of MMP secretion in response to the signals like cytokines, hormones, and cell-cell/cell-ECM contact. These signals result in the activation of either NF-κB, Smad, focal adhesion kinase (FAK) or Wnt. Moreover, epigenetic stabilization of chromatin and mRNAs significantly contribute to MMP expression [90]. Control of MMP expression at the transcriptional level is prevalent in most tissues. Promoters of MMPs have *cis*-elements which favor binding for diverse *trans*-activators like AP-1, polyomavirus enhancer A binding protein-3 (PEA3), Sp-1, β-catenin/Tcf-4, NF-κB and others [91]. Depending on the composition of *cis*-elements there are 3 major categories of MMP promoters which are summarized in **Table 4** [91, 92].

RCT is associated with the upregulation of MMPs especially MMP-1, MMP-8, MMP-13 and gelatinases (MMP-2 and MMP-9) [89, 90]. Our findings from rotator cuff tendon of RCT human and RCT rat models revealed an upregulation of MMP-9 where other MMPs were expressed in basal level [7]. This suggests the potential role of MMP-9 in RCT pathology which proceeds through the alteration of COL1 to COL3 ratio. Among the signals that triggers MMP-9 expression, TGF-β is considered to play a potential role in the pathology of several diseases especially cancer [95]. MMP-9 has been associated with several biological responses like wound healing, inflammation, metastasis, vasculogenesis and development [96]. MMP-9 is secreted by immune cells like neutrophils and macrophages and non-immune cells like fibroblasts which are released upon injury/

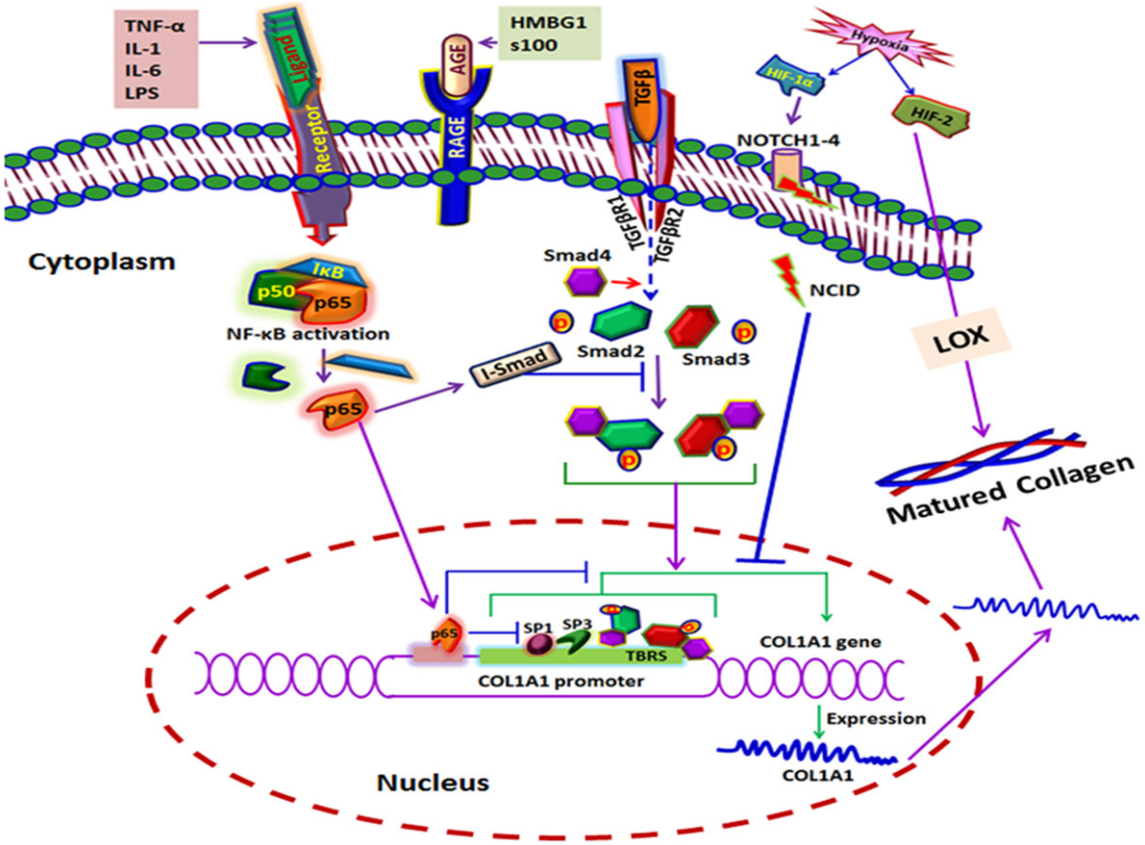

Figure 5. The integration of various signals associated with the regulation of type-1 collagen. Most of the inhibitory signals converge to NF-κB while the stimulatory signals proceed through Smads.

stress. Fibroblasts from most tissue types express MMP-9 when stimulated with IL-1β and TNF-α via ERK1/2 and NF-κB signaling [97].

Apart from NF-κB, PEA3, AP-1, Sp-1 and serum amyloid A-activating factor -1 (SAF-1) also regulate MMP-9 transcription [98]. Reactive oxygen species activates MMP-9 through NF-κB while thrombospondins through AP-1 [99]. TNF-α triggers the expression of MMP-9 by the activation of protein kinase C (PKC) pathway [97]. MMP-9 expression has been associated with overuse tendinopathies of Achilles tendon mostly due to the increase in peripheral circulation of leukocytes [100]. Actual mechanism behind the expression of MMP-9 in shoulder tendon is largely unknown. However, the decrease in COL1 in RC tendon ECM after injury is the major histological hallmark suggesting the COL1 degradation to be the basis for RCT pathology [101].

Tenocyte differentiation is mediated by the transcription factor Scleraxis (Scx) which is induced by TGFβ. On binding with type I and type II transmembrane serine/threonine kinases TGFβ initiates activation and phosphorylation of intracellular effectors (Smad2 and 3, Erk1/2 and p38 MAP kinases). Activation of Smad3 induces Scx upregulation and Mohawk (Mkx). Mkx is required for tendon development and maturation [102]. Apart from these, Smad3 upregulates COL1 and activates healing responses [103]. Moreover, Scx upregulates Tenomodulin (Tnmd) and COL1 which are inevitable for tenocyte proliferation [104]. The mechanism behind the activation of Scx and the execution of downstream signaling warrants more research, especially in regard to RCT.

The integration of various signals associated with the regulation of type-1 collagen is displayed in **Figure 5**.

Summary and future directions

RC tendon is composed of mainly type 1 collagen in which the tendon cells (tenocytes and

tenoblasts) are dispersed. The cell density in normal RC tendon is low and a drastic increase in cellularity has been observed histologically after RCT. This shift in cellularity is thought to be responsible for the pathology. Most of the immune and inflammatory cells were characterized in RCT tendon; however, the possibility of existence of other cellular phenotypes should not be neglected. Several theories exist regarding the pathological basis of tendon ECM; the mechanistic models for explaining such theories are still limited. Understanding the pathological basis of collagen gene alteration and COL1 to COL3 ratio warrants extensive research in regard to tendon molecular biology and genetics. The signals involved and the underlying genomics and proteomics in the regulation of collagen expression in tendons (normal and pathological) are still unknown. The establishment of a tendon matrisome database, tendon genomic database and tendon epigenomic database are needed at this time for complete understanding of RCT pathology. Novel approaches like tendon matrisome remodeling and tendon matrisome engineering should be practiced from *in vitro* to *in vivo* animal models and to be translated to clinical arena. The immunomodulatory approaches to activate the self-healing responses of RC tendons after injury could have promising clinical outcomes. The restoration of tendon ECM and homeostasis of collagen type 1 could help the sufferers to gain the tendon function after injury. Stem cell-based approaches are also appreciated. Limited information on the basic science of tendon biology, lack of proper animal models and control specimens offer hurdles to RC tendon research. In conclusion, the basic research on RC tendon pathology is still in its infancy which warrants multidisciplinary approach involving expertise in surgery, basic sciences, biomaterials sciences and genetics which would unravel the mystery and pave ways to the development of effective translational strategies for RCT management.

Acknowledgements

This work was supported by Creighton University LB692 grant to MFD and LB506 grant to DKA from the State of Nebraska. The research work of DKA is also supported by grants from the National Institutes of Health. The contents of this original research article are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or the State of Nebraska.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Devendra K Agrawal, Department of Clinical and Translational Science, The Peekie Nash Carpenter Endowed Chair in Medicine, Senior Associate Dean for Clinical and Translational Research, CRISS II Room 510, 2500 California Plaza, Omaha, NE 68178, USA. Tel: 402-280-2938; Fax: 402-280-1421; E-mail: dkagr@creighton.edu

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