Chapter 11

Mechanisms of Collagen Network Organization in Response to Tissue/Organ Damage

Takaoki Saneyasu, Saeko Yoshioka and Takao Sakai

Additional information is available at the end of the chapter

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Abstract

Fibrosis is a part of the wound-healing response to tissue damage and characterized by excessive accumulation of mainly type I collagen-containing extracellular matrices (ECMs). Transforming growth factor beta (TGF-β) is a profibrogenic master cytokine responsible for promoting differentiation of tissue-resident fibroblasts into myofibroblasts, upregulation of ECM production, and downregulation of ECM degradation. The formation of ECM is an essential response in wound healing. Fibronectin is an ECM glycoprotein substantially expressed during tissue repair. Based on *in vitro* findings, it has been widely accepted that collagen network organization was exclusively fibronectin matrix dependent. Unexpectedly, our fibronectin conditional knockout mouse models have demonstrated a fibronectin-independent mechanism of collagen fibril formation following injury and identified TGF-β signaling and type V collagen as essential elements for collagen fibrillogenesis. Interestingly, the targeting of the TGF-β signaling alone, as proposed in some recent antifibrotic therapies of chronic fibrotic diseases, is not sufficient to completely prevent liver fibrosis. In this chapter, we focus on the present knowledge of the mechanisms of the collagen network organization following tissue/organ damage and pathological processes of chronic fibrotic diseases.

Keywords: collagen, extracellular matrix, fibrogenesis, fibronectin, TGF-β

Abbreviations:

α-SMA, α-smooth muscle actin; BAPN, β-aminopropionitrile; BMP-1, bone morphogenetic protein-1; CCl_4, carbon tetrachloride; Col I, type I collagen; Col III, type III collagen; Col V, type V collagen; Col XI, type XI collagen; CTGF/CCN2, connective tissue growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; HSC, hepatic stellate cell; IL17R, IL-17A receptor; LAP, latency associated protein; LLC, large latent complex; LOX, lysyl oxidase; LTBP, latent TGF-β-binding protein; MMP, matrix metalloproteinase; OB, obliterative bronchiolitis; SLRPs, small leucine-rich proteoglycans/proteins; TGF-β, transforming growth factor-β; TSP-1, Thrombospondin-1; TRI, TGF-β type I receptor; TRII, TGF-β type II receptor; TMLC, mink lung cell line; PAI-1, plasminogen activator inhibitor-1.

1. Introduction

Cells in virtually all tissues are in contact with organized complexes of structural molecules collectively called the extracellular matrix (ECM). ECM induces a variety of signals that regulate the behavior of cells, such as differentiation, adhesion, and migration, and also fundamental physiological processes such as embryonic development and tissue regeneration and remodeling [1]. As a consequence, tissues or organs keep their normal architecture and homeostasis. Aberrations in signal transduction from the ECM cause chronic degenerative and fibrotic disorders.

Considering the adult tissue/organ remodeling following injury, an important unresolved question is how newly deposited ECM contributes to the critical turning point from normal to abnormal healing. Wound healing is a crucial response to maintain tissue/organ structure and integrity after tissue damage, and also tissue/organ homeostasis [2]. Fibrosis is a part of the wound-healing response that maintains organ structure and integrity following tissue damage. However, excessive fibrosis contributes to a number of diseases. Indeed, fibrosis is the common pathological end result of many chronic inflammatory diseases. Fibrosis is an abnormal extension of the wound-healing process that follows tissue damage, characterized by the excessive accumulation of collagenous ECMs. The hallmark of fibrosis is excessive accumulation of mainly type I collagen (Col I) containing ECMs, and therefore involves both wound-healing and fibrotic processes. Fibrosis is recognized as a major cause of morbidity and mortality in most chronic diseases and chronic graft rejection, and also influences tumor invasion and metastasis. Importantly, a critical event in all fibrotic diseases is the activation of myofibroblast, which are the key mediators of fibrotic tissue remodeling [3].

The clarification of regulatory mechanisms underlying excessive accumulation of ECMs in parenchymal organs such as livers during the development of chronic fibrotic diseases is a critical issue. However, currently, the main barrier to designing novel antifibrotic strategies is due to our insufficient understanding of the mechanisms responsible for ECM-network formations following tissue/organ injury. This gap in knowledge translates to lack of experimental models of repair *in vivo*, including gaps in the understanding of the identity and molecular control of factors and cells participating in the repair processes following injury. In this chapter, we will focus on the present knowledge of the mechanisms of the collagen-network organization following tissue/organ damage and pathological processes of chronic fibrotic diseases.

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2. Molecules critical for collagen network formation

2.1. Fibronectin

Fibronectin, a dimeric glycoprotein, exists in two isoforms: a soluble isoform in plasma (plasma-type fibronectin, produced solely by hepatocytes) and an insoluble isoform in tissue ECM (cellular-type fibronectin, produced by a variety of cells). Both isoforms are generated from a single gene by alternative splicing [4]. Although considerable *in vitro* functional studies have indicated that fibronectin isoforms play key roles in cell differentiation, proliferation, migration, and survival [5, 6], knowledge of the functional identity of each fibronectin isoform in adult tissue remodeling remains loosely defined due to the complexity and the lack of the systems. Indeed, a prominent expression of fibronectin is often observed during adult tissue repair [7]. In response to adult tissue damage, the initial "provisional matrix" formation between plasma-type fibronectin and fibrinogen stabilizes wounded area, which acts as a nidus for subsequent collagen fibrillogenesis [7, 8]. Little insight into the pathophysiological roles of fibronectin has emerged from studies of genetic changes in humans. There are no documented cases of fibronectin-null patients, the nearest condition being familial glomerulonephritis in which there are mutations in the type III modules of fibronectin [9]. While complete fibronectin-null mice show an embryonic lethal phenotype [10], experimental evidence has documented that skin wounds heal normally in mice lacking plasma-type fibronectin [11]; hence, an absolute requirement for fibronectin in response to adult tissue damage has been speculative.

Based on *in vitro* findings, it has been postulated that collagen network formation depends on the fibronectin matrix [12, 13]. It was, therefore, hypothesized that the removal of fibronectin from the *in vivo* system could abolish extensive ECM network formation following tissue damage. To define the functional identity of fibronectin in adult tissue remodeling, we recently established a null condition for both fibronectin isoforms in adult liver (fibronectin(fl/fl)/Mx-Cre+). We have demonstrated an unexpected finding that the lack of fibronectin does not interfere with reconstruction and resolution of collagen fibril organization after the initial stages of liver injury. We have discovered a fibronectin-independent mechanism of collagen fibrillogenesis and identified transforming growth factor beta (TGF-β) signaling and type V collagen (Col V) as essential elements for collagen fibrillogenesis in response to liver injury [14] (further discussed in Section 3).

2.2. Transforming growth factor-β (TGF-β)

Transforming growth factor (TGF)-β is a profibrogenic master cytokine responsible for promoting differentiation of tissue-resident fibroblasts into myofibroblasts, upregulation of ECM production including fibronectin, and downregulation of ECM degradation [15–18]. TGF-β is secreted as a biologically inactive (latent) form, and importantly, the active TGF-β levels do not often correlate with mRNA and protein levels [19]. Indeed, the activation of latent TGF-β occurs independently of transcription [20], and the bioassay to measure active TGF-β levels has been developed using a mink lung cell line (TMLC) stably transfected with a plasminogen activator inhibitor-1 (PAI-1) promoter fused to luciferase [21].

TGF-β is secreted in a biologically inactive (latent) form in a complex (large latent complex [LLC]) with TGF-β latency associated protein (LAP) [22] and latent TGF-β binding proteins (LTBPs) [23]. Prior to the secretion, TGF-β is synthesized as a precursor and forms dimerized complex through disulfide bonds intracellularly. The dimer precursor is proteolytically cleaved by endopeptidase furin to generate LAP and mature TGF-β peptide from the N-terminal and C-terminal portions, respectively [24, 25]. Interestingly, LAP still associates with mature TGF-β noncovalently, termed as small latent complex. *In vitro* studies demonstrate that LAP is required for the secretion of TGF-β from cells [26, 27]. Furthermore, LAP shields the receptor binding epitope of mature TGF-β, indicating that LAP plays an inhibitory role in binding to its receptors [18]. Small latent complex is further associated with a secreted large glycoprotein LTBP via disulfide bonds. These trimolecular complexes (TGF-β, LAP, and LTBP) termed as LLC are formed intracellularly, then secreted and incorporated into ECM. An *in vitro* observation reveals that LLC and free LTBPs are secreted rapidly from cells as early as 30 min after synthesis, whereas small latent complex is secreted slowly [28]. Another study using TGF-β expressing CHO cells shows that only 50% of recombinant TGF-β precursor is secreted at 6 h after radio labeling with [^{35}S] cysteine and [^{35}S] methionine, and the level of its secretion becomes plateau at 20 h [29]. Secreted LLCs deposit in ECM via LTBPs. LTBPs are extracellular multidomain glycoproteins and share homology with fibrillins, which are the major constituents of connective tissue microfibrillar structure [25, 30–32]. Four different isoforms (LBTP 1-4) have been identified, and each isoform includes four 8-cystein domains and numerous epidermal growth factor (EGF)-like motifs [32]. Three isoforms, LTBP-1, 3, and 4, are known to associate with LAP via the third 8-cystein domain, whereas LTBP-2 does not bind to latent TGF-β [32]. In addition, LTBPs interact with extracellular proteins such as fibrillin-1, fibronectin, heparin, and myostatin [32–36]. Indeed, LTBP-1 colocalizes with both fibrillin-1 and fibronectin *in vitro* [33, 36]. Thus, LTBP-1 plays a central role in secreting and anchoring latent TGF-β into ECM (see Section 3).

In response to injury, the conformation of LLC changes and/or TGF-β is released from LAP, resulting that active TGF-β is exposed to its receptor binding site [37]. Indeed, elevated TGF-β bioavailability is frequently observed in chronic fibrotic diseases, and the inhibition of local TGF-β activation can protect against the progression of fibrosis in several adult chronic fibrotic diseases [38–41]. There are several mechanisms of local TGF-β activation, which mediates αvβ6 and αvβ8 integrins, and thrombospondin-1 (TSP-1). Integrin αvβ6 can directly activate latent TGF-β, which depends on an interaction with RGD amino acid sequence of LAP [25]. In response to injury, integrin αvβ6 induces a conformational change (deformation) of LAP via the interaction between αvβ6 and the cytoskeleton [25, 42, 43]. Consequently, such a deformation makes it possible to release active TGF-β from LAP and then cause mature TGF-β to interact with TGF-β type II receptor (TRII) [25, 42, 43]. Interestingly, this activation process is independent of any proteolysis [25]. In contrast, integrin αvβ8-mediated activation of TGF-β is shown to be dependent on membrane type 1-matrix metalloprotease (MT1-MMP, also known as MMP-14) [44]. Furthermore, this activation does not require β8-cytoplasmic domain [44]. These findings indicate that the mechanism of αvβ8-mediated TGF-β activation is clearly different from that of αvβ6. *In vitro* and *in vivo* studies demonstrate that TSP-1 can also activate latent TGF-β [45, 46]. TSP-1 is a matricellular protein prominently expressed in response to tissue damage and plays a role as a transient component of ECM during tissue repair [47]. TSP-1 directly interacts with the LAP [48], and the interaction is supposed to induce a conformational change of LAP, thereby presumably uncover TGF-β receptor binding site [49, 50]. In addition, MMP-2, 9, 13, bone morphogenetic protein (BMP)-1, and serine proteases (plasmin, thrombin, neutrophil elastase, and kallikrein) have been shown to play an important role in TGF-β activation at least *in vitro* [51, 52]. A very recent study reveals plasma kallikrein-dependent TGF-β activation in fibrotic liver in both animal models and patients [53].

Smads are the central as direct downstream modulators in canonical TGF-β signaling [54–56]. Smads consist of three classes: regulatory/receptor-activated (Smad2 and Smad3), coactivating (Smad4), and inhibitory Smads (Smad6 and Smad7). TGF-β binding to TRII initiates the formation of the complex with the TGF-β type I receptor (TRI) and phosphorylation of TRI. Subsequently, they activate TRI phosphorylates of Smad2 and Smad3, and then they form complexes with Smad4. These complexes bind to specific motifs "Smad-binding element" with transcription factors/coactivator such as Ap-1, Sp1, and CBP/p300, and promote the gene expression [54, 57]. Lines of evidence suggest that TGF-β signaling plays a key role in regulating myofibroblast phenotypes and fibrosis in the heart, lungs, liver, kidneys, and skin [3, 54]. For example, TGF-β directly induces the transdifferentiation of fibroblasts into collagen-secreting active myofibroblasts [3, 14, 58], and overexpression of TGF-β results in the induction of hepatocyte apoptosis and liver fibrosis [18, 59, 60]. In contrast, TGF-β1 knockout mice show remarkable (~80%) decrease of collagen accumulation in response to liver injury [58, 61]. Furthermore, Smad3-null mice show reduction in liver fibrosis with decreased myofibroblast activation and ECM production in response to liver injury, whereas the disruption of inhibitory Smad7 results in an enhancement of damage and fibrogenesis in chronic liver injury [7]. We have previously generated adult mice lacking TRII from livers (TGF-βIIR(fl/fl)/Mx-Cre+); because TRII is the exclusive type II receptor for all TGF-β ligands, lack of this receptor abolishes all TGF-mediated signaling in the liver. Knockout livers actually show significantly lower ECM deposition (~46% compared to controls) in carbon tetrachloride (CCl_4)-induced chronic injury, which is accompanied by the decreased expression of myofibroblast marker alpha-smooth muscle actin (α-SMA). These findings indicate that TGF-β signaling is indeed a dominant pathway in the development of liver fibrosis [62]. However, elimination of TGF-β or TRII does not completely prevent the accumulation of Col I in chronic liver injury, and in particular, TRII knockout livers still remain ~46.4% fibrosis compared to wild type [61, 62]. Therefore, these findings clearly indicate the TGF-β-independent mechanism(s) in the development of liver fibrosis (see Section 3). Indeed, we have found that TRII-null livers significantly upregulated connective tissue growth factors (CTGF/CCN2) following chronic liver injury, suggesting that CTGF/CCN2 can be an alternative mediator in liver fibrosis.

As described above, the local activation of latent TGF-β is a critical step in TGF-β-mediated fibrosis [37, 42, 63, 64]. We have discovered that fibronectin-null livers show elevated local TGF-β bioavailability and upregulate Smad signaling in activated hepatic stellate cells (HSCs) following injury [14, 65]. This novel finding implies that fibronectin regulates the balance between active and inactive (latent) TGF-β, which in turn modulates ECM production and remodeling following injury, and consequently retains adult tissue/organ functions. We further describe this important topic in the following section.

2.3. Type I/type V collagen

Collagens are the most abundant scaffolding ECM in tissue/organ stroma and contribute significantly to tissue/organ integrity [66, 67]. The collagen superfamily is large and heterogeneous, and there are at least 28 different collagen types in vertebrates [66, 68]. A collagen molecule consists of three polypeptide chains called α chains, displays a right-handed supercoil, and also has one or more triple helical regions with common sequence repeats, Gly-X-Y [69]. Collagens are divided into six subfamilies (or subgroups) based on their structure, function, and distribution [66, 67]. The collagens that we focus in this review are Col I and Col V and those belong to fibril-forming collagens. Fibril-forming collagens are synthesized as procollagen and three pro α chains fold a triple helical structure from its C-terminus to N-terminus [67]. To form ordered fibrils, both N- and C-propeptides should be cleaved by procollagen N-proteinases/ADAMTS-2, 3, 14, and procollagen C-proteinases/BMP-1/tolloid proteinases, respectively [68]. An exception to this is the case in Col α1(V) chain. Its C-procollagen is cleaved by furin, and its N-terminal is cleaved by BMP-1 [70]. The peptides cleaved by proteinase are called "propeptides" and noncollagenous peptides remain after the enzyme cleavages called "telopeptides". The telopeptides of Col I contain intermolecular crosslinking sites for fibrillogenesis [71]. Fibril-forming collagens form the 64–67 nm regularly repeated striated fibrils [72]. When collagen I molecules form fibrils, there is a unique space in the collagen fibrils termed "gap zone". The gap zone is present between the N-terminus of one molecule and C-terminus of the next in the triple helix-formed collagen fibrils, and the gap zone is suggested to play a role in a variety of molecular interactions, including lysyl oxidase (LOX)-mediated collagen cross-linking [73, 74].

Collagens are essential for tissue-specific macromolecular structure and organizations in the ECM. Indeed, collagen-mediated ECM networks affect many important biological properties such as matrix/tissue stiffness and tissue/organ structure. Collagens participate in numerous physiological processes such as embryonic development, and tissue regeneration and remodeling [75]. Collagen networks provide the biomechanical scaffold for cell attachment and trap of macromolecules, and regulate cell growth and proliferation, and also the shape and structural integrities of cells and tissues [1, 76]. While collagens exist outside of cell and are composed of ECM structures, cells always sense the alterations of their ECMs, produce new ECMs, and/or degrade their ECMs, and consequently, tissues/organs maintain their homeostasis. More importantly, collagens induce intracellular signaling pathways, and it is mediated by cell surface ECM receptor, integrins. Integrins are transmembrane $\alpha\beta$ heterodimeric receptors that mediate organization of focal contacts, actin-containing cytoskeleton, and ECM. Integrins are a major family of cell-surface-adhesion receptors (composed of 18 α-subunits and 8 β-subunits) [77]. The ligation of integrins by adhesive ligands can induce intracellular signaling events ("outside-in" signaling) and intracellular signaling pathways can control binding avidity of integrins for extracellular ligands ("inside-out" signaling) [77]. Interestingly, there is a "cross-talk" between integrins and receptor tyrosine kinases in certain cell types [78–80].

Several unique features of collagens are spotlighted as targets of medical treatment or biomarkers for diagnosis. For examples, oral administration of Col V induces immunologic tolerance to lung allografts and downregulates lung allograft rejection [81]. Another study shows using a specific monoclonal antibody against Col V C-terminal propeptide that, in the liver fibrosis patients, Col V C-terminal propeptide levels released in the serum have a positive correlation with the total amount of collagens deposited in the fibrotic livers. This finding has a potential to be used as diagnostic and potentially prognostic markers in monitoring liver fibrosis [82].

Considering the mechanisms in collagen fibril network organization, two key molecules, Col V and Col XI, are involved in this process. Although both collagens are fibrillar collagens and minor components in tissues/organs, they play an important role in controlling fibril diameter of assembled collagen [83]. Col V [two $\alpha1$(V) and one $\alpha2$(V)] is known to form heterotypic fibrils with Col l [76]. Col V has a conserved multidomain structure, N-terminal domain [73]. Col V controls collagen fibril diameter through both triple helical [84] and N-terminal domains [83, 85]. There is evidence that Col V regulates the fibril diameter *in vitro* [73, 84, 86]: Col I alone formed a broad distribution of relatively large diameter fibrils, while Col V alone formed much thinner nonperiodic fibrils. Interestingly, fibrils formed from Col I in the presence of increasing amounts of Col V displayed a significant decrease in the mean fibril diameter. The variance of the fibril population is also decreased as the percentage of Col V increases. Despite of presence of Col I, deletion of *col 5a1* gene causes embryonic lethal in a mouse model due to a virtual lack of fibril formation in the mesenchyme [87]. The heterozygous mice (*col 5a1* [+/–]) are viable, but show the reduction of fibril number and collagen content in the skin compared to wild-type mice. Furthermore, the abnormal collagen fibrils are observed in the deep dermis of *col 5a1* (+/–) mice; the mutant fibrils display larger diameter and broad distribution, and the diameter is inconsistent along the fibril length. These findings show that col V is essential for Col I fibril assembly.

Col XI [$\alpha1$(XI), $\alpha2$(XI), $\alpha3$(XI)] is known to form heterotypic fibrils with Col II. Col XI α-chains shares structural homology with Col V and appears to have a similar nucleating function [88]. The first half of the human $\alpha1$(V) N-propeptide has 73% homology with the human $\alpha1$(XI) chain [83]. The $\alpha1$ (XI) chain loses its function by chondrodysplasia mutation [89]. Loss of function mutations in the $\alpha1$ (XI) chain leads to the assembly of Col II and Col V fibrils with abnormally large diameters [73, 89]. This interesting observation clearly indicates that Col XI also have a role to regulate the fibril diameter as Col V. To date, very little evidence in Col XI-mediated fibrillogenesis has been identified, and more detailed studies in the functional role of Col XI remain to be elucidated.

Recently, Burlingham et al. have shown an attractive evidence that Col V is involved in immune response to adult human lung disease, obliterative bronchiolitis (OB), which is characterized by narrowing of bronchiole lumens due to the inflammation and fibrosis [90]. Col V is recognized as an antigen by monocyte and presented to $CD4^+$ T cells (called Th-17), and Col V-specific responses are required both IL-17A produced by Th-17 and the monokines

TNF-α and IL-1β, suggesting that IL-17A induced in response to Col V plays a role in fibrosis. Indeed, IL-17A plays a variety of significant roles in neutrophil recruitment, angiogenesis, inflammation, and autoimmune disease [91]. Very recently, Vittal et al. have shown a unique observation about the relationship between Col V and IL-17A [92]. IL-17A can induce epithelial–mesenchymal transition in rat lung epithelial T-antigen negative cells through upregulation of S100A4 and mesenchymal marker α-SMA, and downregulation of epithelial markers ZO-1 and E-cadherin. Mechanistically, IL-17A results in the downregulation of *Smad7*, and upregulation of *TGF*-β and Smad3 activation, clearly indicating that IL-17A is involved in TGF-β pathway. Col V is upregulated by TGF-β during osteogenesis [93], and we have demonstrated TGF-β-induced Col V-mediated *de novo* Col I and Col III network organization even in the absence of fibronectin [14]. Thus, these findings suggest that IL-17A can act as an upstream mediator to regulate the expression of Col V via TGF-β signaling-mediated pathway, and as a consequence, IL-17A could modulate Col V-mediated fibrogenesis. There is another observation showing that IL-17A is involved in liver fibrosis. Mouse primary HSCs are shown to express IL-17A receptor (IL-17R). The treatment of HSC with recombinant IL-17A upregulates α-SMA, collagens, and TGF-β mRNA expression levels. Furthermore, the IL-17RA-null mouse model shows ~50% reduction of liver fibrosis induced by CCl_4 with decreased levels liver damage and inflammations [94], suggesting that IL-17A plays a significant role in HSC transdifferentiation into active myofibroblasts during the development of liver fibrosis. Thus, further analysis of the regulatory mechanisms of Col V by IL-17A could open the new avenue as a drug target for liver fibrosis.

2.4. Other modifying molecules such as lysyl oxidase and small leucine-rich proteoglycans

Collagen contributes significantly to tissue/organ integrity, and collagen cross-linking stiffens the ECM [1]. A recent elegant study has demonstrated that collagen cross-linking leads to cancer progression by enhancing ECM receptor integrin signaling [95, 96]. However, the functional contribution of collagen cross-linking to noncancer pathogenesis remains largely unknown. The LOX family enzymes are copper-dependent amine oxidase and catalyze the post-translational modification of peptidyl lysine to the peptidyl aldehyde, α-aminoadipic-δ-semialdehyde [97]. This chemical change enables the covalent cross-linking of collagen and elastin, resulting in insolubilizing and stabilizing ECM proteins. Collagen cross-linking stiffens the ECM and is accompanied by tissue/organ fibrosis that is mediated by several profibrogenic cytokines [95, 98]. Indeed, LOX inhibitor β-aminopropionitrile (BAPN) reduces organ stiffness following injury and TGF-β1-induced collagen fibril stiffness *in vitro* [65, 99]. Lines of study show that LOX localizes in ECM of several tissue such as skin, aorta, heart, lung, liver, and cartilage [97]. LOX is secreted as inactive proenzyme (proLOX) and then proteolytically cleaved to active enzyme. *In vitro* study shows the possibility that the activation of proLOX occurs on the cell surface in a complex with cellular fibronectin. Indeed, LOX colocalizes well with assembled fibronectin fibrils in cultured fibroblasts and normal human tissues [100]. Furthermore, fibronectin-null mouse embryonic fibroblasts exhibit drastic decrease of the proteolytic processing of proLOX [100], strongly suggesting that fibronectin matrix regulates ECM stiffness via LOX activation.

Small leucine-rich proteoglycans/proteins (SLRPs) such as decorin, biglycan, and fibromodulin are known to contribute significantly to collagen assembly. SLRPs consist of five classes, and almost all SLRPs bind collagen fibrils through their leucine-rich repeat domain [101–103]. Accumulating evidence using SLRP-null mouse models shows that SLRPs regulate the diameter and/or alter structure of collagen fibrils [101–103]. For example, decorin knockout mouse skin exhibits a broader range of collagen fibril diameters [104], and treatment of decorin with decorin-null skin fibroblasts results in the formation of more uniform collagen fibrils [105]. Fibromodulin-deficient tail tendons exhibit thinner collagen fibrils compared to wild-type controls [106, 107]. A dynamic modulus in biglycan-null tendons is significantly increased compared to wild-type tendons [108]. The elasticity of collagen fiber networks in cultured decorin-siRNA-transfected mouse NIH3T3 fibroblasts is declined during the incubation period, whereas it remains unchanged in untransfected cells. It is therefore likely that SLRPs could regulate the physiological properties of ECM (e.g., mechanical strength).

3. Possible mechanisms of collagen network formation

As described in Section 2.1, it has been believed that collagen-network formation depends on the fibronectin matrix in culture [12, 13]. Indeed, the prominent expression of fibronectin is observed during adult tissue repair [11, 109]. Another line of evidence shows that TGF-β plays a central role as a profibrogenic cytokine in the accumulation of ECMs, including fibronectin. Therefore, it would be possible that TGF-β-induced ECM accumulation is dependent on fibronectin. However, to date, fibronectin/TGF-β interdependence in the fibrogenic response to tissue damage has not yet been addressed. Furthermore, it remains to be elucidated how ECM remodeling by myofibroblasts results in changes in mechanical tension and supports the activation of pathogenic signaling pathways during the development of chronic fibrotic diseases. We hypothesized that the removal of fibronectin or TGF-β signaling *in vivo* could prevent extensive ECM network formation following tissue damage. To define the functional identity of fibronectin and TGF-β signaling in adult tissue remodeling, we recently established two animal models lacking fibronectin (both isoforms) or TRII, respectively, in adult liver [14, 62]. Our new findings suggest fibronectin-/TGF-β-independent mechanisms are involved in the development of liver fibrosis.

3.1. Fibronectin-dependent assembly

3.1.1. Fibronectin matrix assembly

Fibronectin matrix assembly consists of multistep process (**Figure 1**) [110–113]. Importantly, fibronectin assembly is cell dependent; binding of fibronectin to cell surface and cellular contractility are required. In the first step, fibronectin binds to cell surface, and integrin plays an important role. Integrins $\alpha5\beta1$ and $\alpha v\beta3$ are characterized as fibronectin receptors [114]. Lines of evidence show that $\alpha5\beta1$ is a primary receptor in fibronectin matrix assembly, whereas $\alpha v\beta3$ dominates the formation of focal contacts [115–117]. However, the binding of fibronectin to the cell is not sufficient for fibronectin assembly. A critical step of this assembly is considered to be the cell-driven exposure of cryptic site for self-association in fibronectin. Although one mechanism for exposing the cryptic site could be conformational changes induced by fibronectin binding to integrin, cellular contractility is necessary for fibronectin fibrillogenesis [88, 111, 118]. Indeed, loss of cellular contractility by RhoA inhibitor prevents fibronectin matrix formation [119]. Furthermore, recent study demonstrates that β1 cytoplasmic domain modulates fibronectin assembly via recruitment of cytoplasmic adaptor protein talin, which links integrin to the actin cytoskeleton [120, 121]. Thus, integrin-mediated association of fibronectin with cytoskeleton is important for fibronectin assembly.

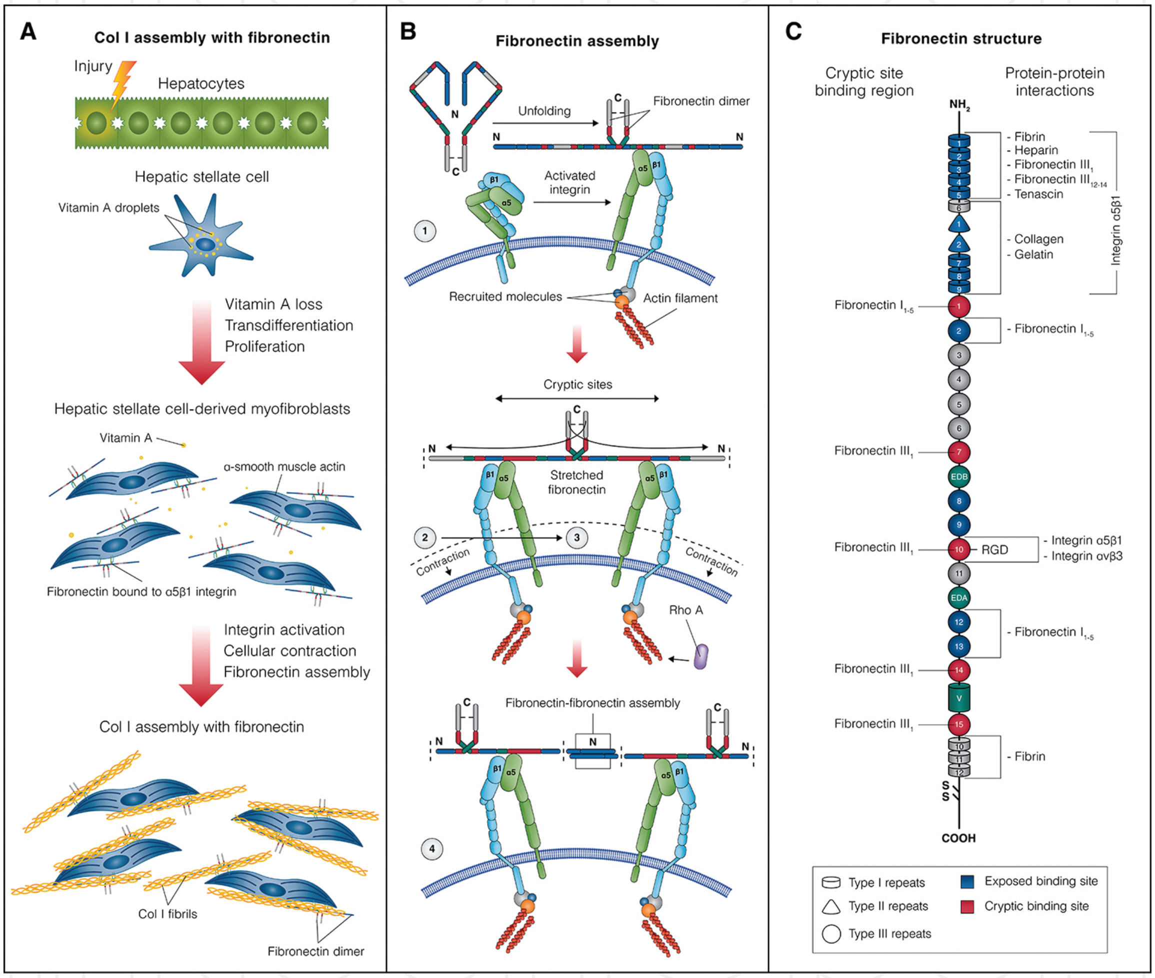

Figure 1. Proposed mechanism of fibronectin-dependent collagen assembly. (A) Type I collagen assembly with fibronectin. (B) Fibronectin assembly. ①Fibronectin binding to activated integrin; ②conformational changes of fibronectin cryptic site by cellular contraction; ③exposure of fibronectin cryptic site, resulting in acquiring ability to associate with other fibronectin molecules; and ④formation and extension of fibronectin fibril assembly. (C) Fibronectin structure. See details in the text.

3.1.2. Collagen assembly with fibronectin matrix

Fibronectin contains collagen-binding domain [122] and directly binds to collagens. *In vitro* studies have shown an extensive codistribution of fibronectin and Col I/III (**Figure 1**) [12, 123]. A very recent study using fibroblasts demonstrates that type I collagen fibrils preferentially colocalize with more-relaxed fibronectin fibrils in the ECM *in vitro* [124]. Fibronectin-null embryonic fibroblastic cells cannot organize collagen fibril networks *in vitro*, but they can form collagen networks when fibronectin is exogenously added [123]. Interestingly, collagen-binding integrins α2β1 and α11β1 are not required for collagen polymerization in fibronectin-null embryonic fibroblastic cells when cultured with exogenous fibronectin [12]. Furthermore, integrin β1-null line GD25 cells, which express fibronectin and its receptor αvβ3, contract collagen gel more forcefully than integrin α2- and β1-transfected GD25 cells that contract collagen gel via α2β1 but not αvβ3 [125]. Other studies show that fibronectin polymerization stimulates collagen gel contraction [126] and that the disruption of fibronectin–collagen association inhibits this contraction [13]. These findings demonstrate that fibronectin matrix is required for collagen assembly and enhances ECM contraction of cells.

3.1.3. Initial incorporation of latent TGF-β complex

Growing evidence suggests a mechanism by which fibronectin plays a role in the initial incorporation of latent TGF-β complex into ECM [18, 33]. LTBP-1 associates with not only fibrillin-1 but also fibronectin [33, 36]. Fibronectin-null fibroblasts fail to incorporate LTBP into ECM [33], and minimally activate latent TGF-β [127]. Furthermore, cells lacking fibronectin receptor integrin α5β1 show defective activity of latent TGF-β by αvβ6 [127]. These findings suggest that fibronectin regulates latent TGF-β activation via deposition of latent TGF-β into the matrix.

3.2. Fibronectin-independent collagen assembly

As described above, based on *in vitro* findings, it has been postulated that collagen network organization and assembly depends on the fibronectin matrix in culture [12, 13]. However, the contribution of fibronectin to these processes remains to be defined *in vivo*. We therefore investigated whether fibronectin is a suitable molecular target for ameliorating the fibrogenic response to liver injury. Since mice with complete inactivation of fibronectin gene die at an early embryonic stage [10], we generated conditional fibronectin-floxed and liver-specific adult fibronectin-null mice (lacking both plasma and cellular isoforms of fibronectin) using *Cre-loxP* technology [11, 14], investigated their phenotypes, and have demonstrated fibronectin-independent mechanisms for collagen network formation following liver injury.

3.2.1. TGF-β and Col V-mediated collagen assembly in fibronectin-null liver

The adult mouse model lacking fibronectin shows no abnormalities in anatomical and histological analyses of the liver and hepatic biochemical markers under standard laboratory conditions. Unexpectedly, the lack of fibronectin did not interfere with the reconstruction of collagen fibril organization in response to both acute liver and chronic liver injuries up to 8 weeks induced by CCl_4 [14]. Fibronectin-null livers show significant increased HSC activation with elevated Smad signaling following injury. To determine whether TGF-β is involved in Col III/I collagen network formation in the absence of fibronectin, we further assessed which factors that regulate activated HSC phenotypes were involved in collagen fibrillogenesis. We have identified TGF-β1-induced Col V as a novel and essential element for Col I/III fibrogenesis in hepatic stellate cells (**Figure 2**). Thus, our study provides compelling evidence that collagen fibrillogenesis in response to adult tissue/organ damage is mediated by both fibronectin and type V collagen.

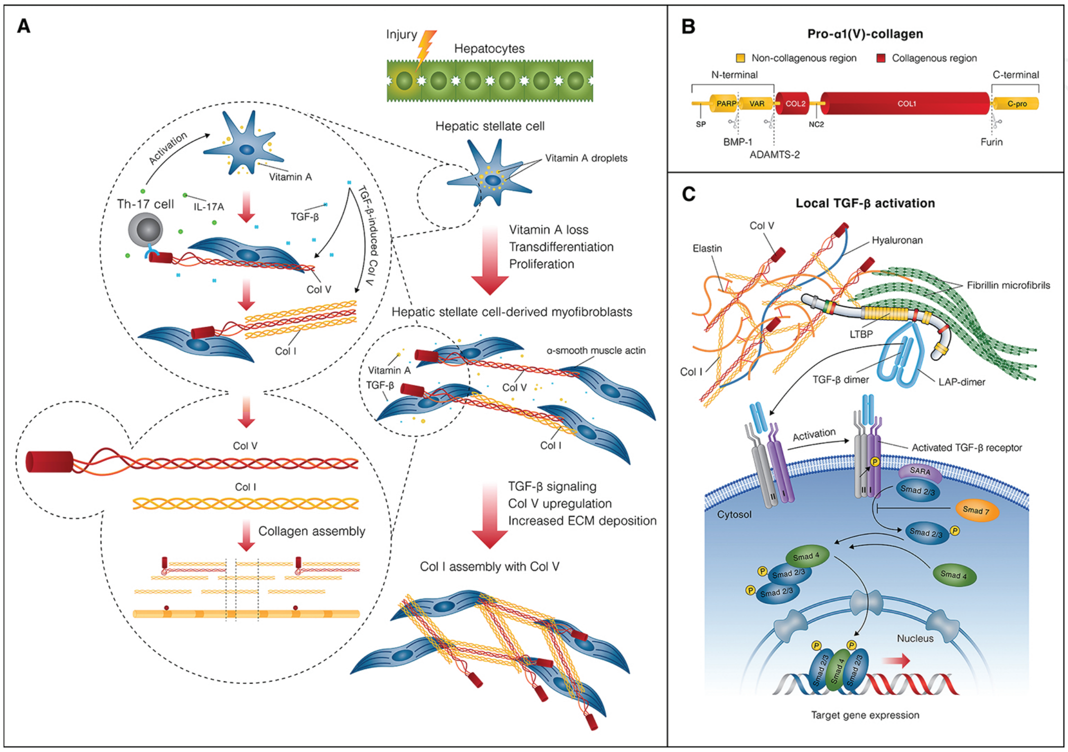

Figure 2. Proposed mechanism of fibronectin-independent collagen assembly mediated by tissue growth factor beta (TGF-β) signaling and type V collagen (Col V). (A) Type I collagen assembly with Col V. (B) Pro-α1(V) collagen structure. PARP, proline- and arginine-rich domain; VAR, variable domain. (C) Local TGF-β activation. See details in the text.

Interestingly, type V collagen–mediated Col III/I fibril assembly following liver injury seems to be specific for adult HSCs because TGF-β1 supports neither Col V nor Col III/I fibril assembly in fibronectin-null embryonic fibroblasts *in vitro* [14]. Furthermore, fibronectin-null livers show substantial depositions of LTBP-1, -3, and -4 with fibrillar structures in the ECM following both acute and chronic liver injuries [14, 65], whereas fibronectin-null embryonic fibroblasts fail to incorporate LTBP-1 into ECM *in vitro* [33]. Therefore, further studies for mechanisms underlying Col V-mediated collagen network formation remain to be elucidated, i.e., the phenotypic differences among myofibroblasts, and the contribution to adult tissue/organ remodeling following injury.

3.2.2. Elevated collagen matrix stiffness in advanced stage of liver fibrosis/cirrhosis in fibronectin-null liver

We showed that collagen network organization can be formed even without fibronectin in response to liver injury [14]. However, it remains unknown whether the initial deposition of fibronectin could contribute to the turning point from normal healing to chronic fibrotic disorders. Furthermore, it remains to be elucidated how ECM remodeling by myofibroblasts affects mechanical tension and supports the activation of pathogenic signaling during the development of chronic fibrotic diseases. We therefore have further investigated whether fibronectin could be a suitable target for ameliorating fibrosis during advanced stages of chronic liver injury [65]. Fibronectin-null livers have exhibited constitutively elevated local TGF-β activity, induced more myofibroblast phenotypes, and accumulated highly disorganized/diffused collagenous ECM networks during chronic liver fibrogenesis induced by CCl_4. The deposition and network formation of Col V are also significantly increased. Consequently, fibronectin-null livers have led to more extensive liver cirrhosis, which is accompanied by significant increased liver matrix stiffness and deteriorated hepatic functions. Mechanistically, fibronectin-null livers have shown elevated LOX expressions, and a significant amount of active LOX is released in fibronectin-null hepatic stellate cells in response to TGF-β1. Furthermore, treatment of fibronectin in fibronectin-null stellate cells recovers collagen fibril stiffness to wild-type levels [65].

We propose that there are the functional links between fibronectin-mediated control of TGF-β bioavailability and collagen fibril stiffness regulated by LOX. All these novel findings strongly suggest that locally activated TGF-β signaling and Col V are essential elements for collagen fibrogenesis without fibronectin in adult tissue remodeling. Although the contribution of Col V-nucleated Col I fibrogenesis in adult tissues is largely undetermined, our finding raises the hypothesis that the accumulation of Col V-mediated ECMs during persistent chronic damage could influence a formation of disorganized ECM architecture.

3.3. TGF-β-independent collagen assembly

3.3.1. CTGF-mediated collagen assembly

TGF-β signaling is the dominant pathway of ECM productions in HSCs [58]. To address whether the elimination of this signaling is sufficient to prevent liver fibrosis, we have generated adult liver-specific TRII-null mice (TGFβIIR[flox/flox]/Mx-Cre+) [62]. Actually, this mutant liver exhibits a significant decrease of ECM deposition and α-SMA expression in CCl_4-induced chronic liver injury. However, elimination of TRII does not completely prevent the collagen accumulation in chronic liver injury, and TRII-null livers still remain ~46.4% fibrosis compared to wild type. Furthermore, we have found that matricellular protein CTGF/CCN2 expression is significantly upregulated (1.94-fold compared to wild type) in mutant livers following chronic liver injury. Therefore, these findings clearly indicate that TGF-β-independent mechanisms play an alternative role in developing liver fibrosis. Since CTGF/CCN2 synergizes with the action of TGF-β, CTGF/CCN2 is considered to act as a TGF-β downstream modulator [128]. Accompanied by the elevated TGF-β activity, CTGF/CCN2 expression is upregulated in several fibrotic tissues, including kidney, lung, heart, liver, pancreas, bowel, and skin [128, 129]. A recent study has demonstrated that overexpression of CTGF/CCN2 in fibroblasts alone is sufficient to cause spontaneous multiorgan fibrosis *in vivo* and that this signal pathway does not involve canonical TGF-β-Smad signaling *in vitro*.

However, the liver does not show significant fibrosis [130]. We have found that TRII-null livers in chronic injury show elevated expression of CTGF/CCN2 in spite of a lack of TGF-β signaling, indicating CTGF/CCN2 as a potent mediator in liver fibrosis [62]. We propose two hits, induction of CTGF/CCN2 and adult tissue/organ injury, for the progression of liver fibrosis. In fibrotic livers, CTGF/CCN2 is known to be synthesized in a diversity of cells such as hepatocytes, myofibroblasts (activated HSCs), endothelial cells, proliferating bile duct epithelial cells, and inflammatory cells [128]. Thus, it remains to be elucidated to identify cellular contribution and mechanisms underlying CTGF/CCN2 production in the progression of tissue/organ fibrosis.

4. Perspective

Novel findings obtained imply that fibronectin regulates the balance between active and inactive (latent) TGF-β, which in turn modulates ECM production and remodeling following injury, and consequently retains adult tissue/organ functions. This regulatory mechanism by fibronectin could be translated for a potential therapeutic target in a broader variety of chronic fibrotic diseases. It is obvious that fibronectin matrix networks play crucial roles in many important biological events and in adult homeostasis. Therefore, the regulation of TGF-β bioavailability by fibronectin with retaining fibronectin matrix networks would be an essential element for long-term antifibrotic strategies in chronic fibrotic diseases to preserve tissue/organ function and homeostasis.

Considering the adult tissue/organ remodeling following injury, important unresolved questions are (1) what is the trigger for fibrosis resolution (regression); and (2) how fibrosis resolution proceeds. Quiescent HSCs are adipocyte-like (vitamin A stock) cells expressing marker genes such as PPARγ, SREBP-1c, and leptin, whereas activated HSCs are proliferative myofibroblasts expressing myogenic marker α-SMA, c-myb, and MEF-2 [131]. CCl_4-induced experimental liver injury models have suggested that elimination of activated HSCs by apoptosis [132] or senescence [133] is a key step in the onset of fibrosis regression [134]. Growing evidence demonstrates that activated HSCs are reverted to quiescent-like state both *in vitro* [135, 136] and *in vivo* [137, 138], and a very recent *in vitro* study using human primary HSCs reveals EGF, fibroblast growth factor 2, fatty acids, and retinol as potential factors in activated HSCs to reverse quiescent-like phenotypes [139]. Interestingly, reverted HSCs show more rapid reactivation into myofibroblasts in response to TGF-β than quiescent HSCs [137]. It is therefore likely that reverted HSCs may not fully revert to a quiescent state [134]. A recent elegant study demonstrates that a history of liver injury is transmitted to offspring via epigenetic modification of PPARγ and TGF-β genes in rats, and consequently, healing response to hepatic injury is suppressed in offspring of CCl_4-injured group compared to injury-inexperienced group [140]. Nevertheless, the clarification of molecular mechanisms underlying excessive accumulation of collagenous ECM during the development of chronic fibrotic diseases could translate basic antifibrotic research into improved clinical therapeutic approaches, which will have a significant benefit in public health impact. Thus, an establishment of novel models/systems, e.g., in which we enable to analyze global alterations of signaling cascades/metabolisms or epigenetics, would be crucial for challenging these complicated mechanisms in chronic fibrotic diseases.

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Conflict of interest

The authors disclose no conflicts.

Author details

Takaoki Saneyasu, Saeko Yoshioka and Takao Sakai*

*Address all correspondence to: sakait@liverpool.ac.uk

MRC Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, Liverpool, UK

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